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ERRATA AND AUTHORS' EMENDATIONS

Pages 51, 53, 55, and 57, the running head "Heterothallism and Hybridization in Wheat" should be "Heterothallism and Hybridisation in Tilletia."

Page 114, eleventh line from bottom, "0.14N H_2SO_4" should be "0.0714N H_2SO_4."

Page 135, line 12, delete "of" between "form" and "malady."

Page 207, line 4, "(31)" should be "(24)."

Page 246, Table 1, last column, "902" should be "290."

Page 265, under Literature Cited, reference (3), "Collision" should be "Collison."

Page 273, sixteenth line from bottom, insert "provide" between "to" and "2.25."

Page 352, line 5, delete "in 1928."

Page 374, heading of Table 2, "22°" should be "25°."

Page 376, line 15, "Table 2" should be "Table 1."

Page 378, legend for Figure 2, second line, insert "(B)" between "latter" and "after."

Page 420, Figure 3, the arrow opposite January should point to the first solid line instead of to the first broken line.

Page 526, Table 3, heading of fourth and ninth columns, "C.DCA" should be "C.DBA."

Page 526, Table 3, eighth column, last figure, "60.00" should be "70.00."

Page 563, Table 2, first column, "-8-4" should be "4-8-4."

Page 569, footnote 11, "Cummings" should be "Cumings" and "Lewis" should be "Servis."

Page 638, line 6, delete horizontal bar where it first appears over w and also over z, so as to make formula read:

$$bS(w^2w-w\overline{w})+cS(w^2-\overline{w}^2)^2=S(zw^2-z\overline{w}^2)$$

Page 638, line 20, delete bar over last z.

Page 639, line 30, insert closing parenthesis between "w" and brace, so as to make formula read:

$$S\{(z-\overline{z})-b(w-\overline{w})\}^2$$

Page 639, line 32, the square (²) at the end of the formula should be placed between the parenthesis and the brace, so as to make the formula read:

$$S\{(z-\overline{z})^2-2b\,(zw-z\overline{w})+b^2(w-\overline{w})^2\}$$

Page 684, Table 3, under Calorimeter Method, columns 5 and 6, "+" should be "+."

Page 696, line 16, last word, "Moelleriella" should be "Moelleriella."

Page 713, Table 2, column 3, only one test was made for olive organism (Petri's Italian strain), using Witte's peptone and indigo carmine. The two paragraphs in question should therefore be combined into one.

Page 849, third line from bottom, "unshelled" should be "shelled." The key number should be "N.C.-27."

Page 861, footnote 3, "Vargleichende" should be "Vergleichende."

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VOL. 44 WASHINGTON, D. C., JANUARY 1, 1932 No. 1

A STUDY OF THE PHYSIOLOGIC FORMS OF KERNEL SMUT (SPHACELOTHECA SORGHI) OF SORGHUM [1]

By L. E. MELCHERS, *Plant Pathologist, Kansas Agricultural Experiment Station, and Agent, Division of Cereal Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture;* C. H. FICKE, *Junior Pathologist;* and C. O. JOHNSTON, *Associate Pathologist, Division of Cereal Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture*

INTRODUCTION

Studies have been made at the Kansas Agricultural Experiment Station over a period of 14 years (1916 to 1930) on the kernel smut (*Sphacelotheca sorghi* (Link) Clinton) of sorghum (*Sorghum vulgare* Pers.). These studies have included varietal resistance (*7*),[2] improved methods for control (*2, 5*), physiologic specialization, cultural studies on artificial media (*6, 1*), and the mode of inheritance of smut resistance in sorghums (*8*). In varietal studies by Reed and Melchers (*7*) over a period of seven years at Manhattan, Kans., Brooklyn, N. Y., Columbia, Mo., and at the Arlington Experiment Farm, Rosslyn, Va., certain varieties of durra, all varieties of milo, selections of feterita, darso, Dwarf hegari, and Sudan corn included in the tests were highly resistant to or immune from the common kernel smut, *S. sorghi*. Following this study, Tisdale, Melchers, and Clemmer (*9*) called attention to the fact that pure lines of milo, hegari, and feterita were attacked by kernel smut. They believed the smut on milo and hegari to be a distinct physiologic form of kernel smut, but were uncertain whether it was a strain of *S. sorghi* or a hybrid fungus between *S. sorghi* and the loose kernel smut, *S. cruenta* (Kuehn) Potter, as the fungus had characteristics of each. The kernel smut found on the feterita was considered to be a distinct form.

Kulkarni (*3*) in 1921 made some interesting observations in India which are worthy of consideration. He obtained seed of Dwarf Yellow milo from the United States Department of Agriculture and after inoculation with spores of *Sphacelotheca sorghi* and *S. cruenta* sowed it on the agricultural college farm at Poona, India. He obtained 635 heads, of which 3 were infected by *S. sorghi* (0.47 per cent infection) and 50 by *S. cruenta* (7.9 per cent infection). Although Kulkarni did not suspect physiologic forms, he did call attention to the fact that it appears very evident that milo can not be regarded as immune from *S. sorghi* as all literature up to that time indicated, and that it must be regarded as decidedly susceptible to *S. cruenta*. In the light of present knowledge on the subject, it is evident that this investigator had more than one physiologic form of sorghum smut.

Recently Melchers, Ficke, and Johnston (*6*) and Ficke and Johnston (*1*) have shown that at least three physiologic forms of *Sphacelotheca*

[1] Received for publication June 30, 1931; issued February, 1932. Paper No. 306 from the Department of Botany and Plant Pathology, Kansas State Agricultural College, in cooperation with the Division of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture.

[2] Reference is made by number (italic) to Literature Cited, p. 11.

sorghi occur, and these are specifically designated by them as forms 1, 2, and 3. The former (*6*) have shown that form 1 does not attack milo, hegari, and feterita; that form 2 attacks milo and hegari but not feterita; and that form 3 attacks feterita and certain feterita hybrids, but not milo.

In the present paper, data are presented on the reaction of a large number of varieties, hybrids, and selections of sorghum to five physiologic forms of *Sphacelotheca sorghi*, including the three above mentioned. These forms are determined by the reaction of varieties of sorghum having different degrees of resistance, as well as by certain morphologic differences in the strains of the smut fungus itself.

It is not the purpose of this paper to review all the literature on physiologic specialization in the fungi. This is a common phenomenon in the rusts, smuts, and certain other fungi and undoubtedly will eventually be found to be true of still other fungi, being of more significance and economic importance in some species than in others. Those papers on physiologic specialization that deal directly with the subject matter herein presented are cited.

MATERIALS AND METHODS

Seed of the several varieties of sorghum was obtained from different sources and was collected over a period of years.[3] As sorghums hybridize so readily, a sufficient number of heads of each variety were self-pollinated each year to assure pure seed for these experiments. All seed was disinfected in a formaldehyde solution (1 to 320) for 30 minutes, and washed and dried before sowing. A measured quantity of spores of each of the several forms of smut was mixed with weighed quantities of seed. The seed and spores were then shaken together until the seed was completely covered with spores. This was done for a complete series of sorghums for each physiologic form of smut. The optimum conditions for kernel-smut infection at Manhattan, Kans., have been found, over a period of years, to be those existing at the optimum time for sorghum planting or a little earlier. This is about May 15 at Manhattan, a time when soil temperatures range between 16° and 23° C. Somewhat lower or higher temperatures, however, do not prevent infection. If sufficient soil moisture is present for germination of sorghum seed, it is suitable for the germination of the smut spores and infection. Soil-moisture determinations made on different dates of planting under field conditions from 1926 to 1928, inclusive, show that the soil-moisture range at which heavy infection may occur is very great. Thus soil-moisture percentages from 15 to 38 per cent, by dry weight, have proved favorable for heavy smut infection when the soil temperature was within the range previously stated.

The method of planting, number of plants per row, and manner of taking the smut notes have been described in previous publications (*2*, *7*). The milo kernel smut used in these investigations is a pure strain of the material occurring on milo, originally collected in New Mexico and Kansas, and used by Tisdale, Melchers, and Clemmer in their studies (*9*). The feterita kernel smut also is a pure strain of the material that came originally from Texas and was used by these investigators.

[3] The writers are indebted to J. H. Parker and A. F. Swanson, of the Kansas Agricultural Experiment Station and the Fort Hays Branch Experiment Station, respectively, for the seed of many of the varieties.

RESULTS

STUDIES ON PHYSIOLOGIC SPECIALIZATION OF SPHACELOTHECA SORGHI

Three years' data (1927 to 1929) have been obtained on physiologic specialization of *Sphacelotheca sorghi* at Manhattan, Kans. Eighty varieties, selections, and hybrids of the various groups of sorghums have been grown to determine their reaction to three forms of smut. Data obtained in 1929 suggest the presence of two additional physiologic forms, making a total of five. (See Table 3.) These physiologic forms of *S. sorghi* have been segregated on the basis of (1) the reaction of differential hosts, (2) cultural characteristics on artificial media, and (3) differences in the fragility, size, and color of the sori.

Table 1 gives the data on varietal reaction to form 1, designated as the common kernel smut; to form 2, the milo kernel smut; and to form 3, the feterita kernel smut. Blanks denote that no smut of that form or seed of the variety was available.

TABLE 1.—*Percentage of smutted heads in sorghums inoculated with physiologic forms of kernel smut (Sphacelotheca sorghi) from kafir, milo, and feterita, at Manhattan, Kans., 1927, 1928, and 1929*

Group and variety, selection, or hybrid	Accession No.[a]	Percentage of heads smutted after inoculation with physiologic form indicated								
		1927			1928			1929		
		Form 1 (kafir)	Form 2 (milo)	Form 3 (feterita)	Form 1 (kafir)	Form 2 (milo)	Form 3 (feterita)	Form 1 (kafir)	Form 2 (milo)	Form 3 (feterita)
Sorgo:										
Dwarf Sumac	K. B. 2576	46.3			32.1	23.7	27.3	46.9	33.1	31.7
Standard Sumac	K. B. 2902							40.4	13.9	64.5
Red Amber	K. B. 2504	26.5	10.1		32.3	19.8	15.1	17.1	17.1	11.6
Kansas Orange	K. B. 2560	47.8	23.7		20.8	17.5	34.1	26.1	25.6	38.4
Leoti Red	F. C. I. 6610	34.7			53.2	25.5	11.3	72.2	31.3	30.1
Freed	K. B. 2519	14.6			32.3	34.7	44.3	9.9	20.0	30.4
Weskan	K. B. 2522				53.5	50.0	37.6	16.7	26.1	33.1
Honey	K. B. 2874				61.2	49.7	44.3	38.6	34.9	24.2
"Japanese Honey Drip"	K. B. 2876				7.5	7.9	8.1	29.3	25.9	27.9
Atlas selection No. 95	K. B. 2877				96.2	68.9	34.1	20.3	33.1	34.3
Atlas selection No. 100	K. B. 2878				84.1	53.1	50.6	11.1	35.4	20.8
Kafir:										
Reed	K. B. 2502	23.8			38.5	31.8	53.1	23.1	16.5	23.0
Pink	K. B. 2506	36.7	49.1	50.3	65.8	37.2	33.3	18.8	50.0	27.8
Pink selection	K. B. 2546	67.9			68.3	44.4	53.4	25.8	37.8	21.9
Pink	F. C. I. 9091				3.0	0	.4	15.8	35.6	39.5
Early Pink	K. B. 2824				21.4	1.6	2.8	29.2	33.3	27.2
Sunrise	K. B. 2523	70.8			54.8	36.4	39.6	23.3	20.2	23.8
Blackhull	K. B. 2535	40.2	16.7		62.9	28.0	33.7	8.4	24.0	13.6
Western Blackhull	K. B. 27102	2.2			53.2	44.2	38.1	9.5	32.7	25.7
Dawn	K. B. 2538	20.9			57.1	45.3	40.3	23.9	26.8	20.0
Dawn selection	H. C. 2421	68.6	33.3		51.0	39.4	32.7	12.5	24.4	16.2
Club	H. C. 281				46.5	27.1	35.1	15.8	19.0	10.1
Red	K. B. 2545	47.5			89.4	26.0	40.6	38.4	25.2	30.8
Milo:										
Dwarf Yellow	C. I. 332	0	10.4		0	10.7	0	0	13.2	0
Dwarf Yellow selection	K. B. 2511	0	6.3	0	0	16.1	0			
Do	K. B. 2515	0	22.8		0	11.3	0	0	18.0	0
Standard Yellow	C. I. 234		10.4		0	11.8	0	0	8.2	0
Dwarf White	F. C. I. 8927	0	0	0	0	13.4	0	0	7.5	0
Standard White	C. I. 352		16.8		0	9.3	0	0	7.9	0
Cream	K. B. 2569	0	12.5	0	0	10.5	0	0	12.5	0
Fargo Straightneck	C. I. 809	25.0	3.2	0	19.2	4.4	0	3.3	25.3	0
"Dwarf Straightneck"	K. B. 2844				0	2.6	0	0	25.6	1.1
"Erect"	K. B. 2845				0	19.7	0	0	32.6	0

[a] The letters C. I., F. C. I., S. P. I., H. C., and K. B. indicate accession numbers, respectively, of the Division of Cereal Crops and Diseases, Division of Forage Crops and Diseases, and Division of Foreign Plant Introduction, all of the Bureau of Plant Industry, U. S. Department of Agriculture, and of the Fort Hays branch experiment station and the department of botany and plant pathology, both of the Kansas Agricultural Experiment Station.

TABLE 1.—*Percentage of smutted heads in sorghums inoculated with physiologic forms of kernel smut (Sphacelotheca sorghi) from kafir, milo, and feterita, at Manhattan, Kans., 1927, 1928, and 1929*—Continued

Group and variety, selection, or hybrid	Accession No.	Percentage of heads smutted after inoculation with physiologic form indicated								
		1927			1928			1929		
		Form 1 (kafir)	Form 2 (milo)	Form 3 (feterita)	Form 1 (kafir)	Form 2 (milo)	Form 3 (feterita)	Form 1 (kafir)	Form 2 (milo)	Form 3 (feterita)
Feterita:										
Feterita selection	C. I. 182–1	0	0	0	0	0	0	0	0	2.1
Do	K. B. 2563	0	0	0	0	4.3	0	0	0	0
Feterita	S. P. I. 51989	0	0	24.8	0	0	17.9	0	0	2.6
Spur	K. B. 2540	0	0	.....	0	0	0	0	0	0
Red Leaf selection	K. B. 2543	0	.....	.....	.....	0	4.9	0	0	3.9
Do	K. B. 2544	.....	.....	.....	0	0	3.4	3.3	0	4.5
Hybrid Dwarf No. 6	K. B. 2820	.....	.....	.....	0	0	.6	2.5	0	3.2
Hegari:										
Hegari selection	K. B. 2518	0	2.9	0	0	8.6	0	0	1.0	0
Hegari	K. B. 2537	.....	.....	.....	0	9.0	0	0	13.6	0
Hybrids:										
Red Amber×feterita selection	K. B. 2501	0	8.2	0	0	20.5	0	0	3.4	0
Do	K. B. 2509	0	0	0	0	0	0	0	0	0
Do	K. B. 2513	0	0	0	0	3.8	0	0	0	0
Do	K. B. 2552	0	12.3	1.0	1.5	8.1	.5	.....	.....	0
Do	K. B. 2562	0	0	0	0	0	0	0	0	0
Do	K. B. 2567	0	10.4	0	0	0	0	0	2.5	0
Do	K. B. 2570	21.5	0	15.9	25.9	0	18.1	13.8	0	21.3
Do	K. B. 2573	0	0	0	0	0	0	0	0	0
Kafir×feterita	F. C. I. 8920	.....	.....	.....	5.6	2.2	3.2	0	4.2	1.6
Do	H. C. 2423	0	0	.....	0	0	18.4	0	.....	.....
Feterita×kafir	F. C. I. 8917	.....	.....	.....	0	0	34.4	0	0	2.4
Kafir×milo 26–3–1–1	K. B. 2561	0	34.5	10.9	0	37.0	21.8	0	26.3	13.0
Kafir×milo 38–1–2–1	K. B. 2679	42.1	0	6.3	44.3	39.3	16.4	13.2	40.8	22.5
Dwarf Yellow milo×Pink kafir.	H. C. 244	52.4	.....	.....	51.1	52.1	51.5	0	20.3	16.3
Do	H. C. 257	0	.....	.....	0	26.1	12.3	0	12.8	5.6
Do	H. C. 2510	13.0	17.7	.....	37.4	13.2	0	6.3	5.6	4.3
Kansas Orange×Dwarf Yellow milo selection 1.	K. B. 2680	.....	.....	.....	60.4	34.5	8.5	9.4	45.0	39.6
Kansas Orange×Dwarf Yellow selection 2.	K. B. 2681	.....	.....	.....	0	5.3	0	0	0	0
Kansas Orange×Dwarf Yellow selection 6.	K. B. 2682	0	.....	.....	62.1	56.4	48.0	23.0	41.1	36.8
Pink kafir×Freed sorgo	K. B. 2798	56.0	.....	.....	50.8	26.7	53.2	23.9	43.2	45.3
Milo×feterita	F. C. I. 8926	0	0	5.2	0	.4	7.9	0	0	0
Blackhull×Sourless	K. B. 2505	56.0	.....	.....	60.7	62.9	52.7	9.4	.....	.....
Broomcorn:										
Acme (dwarf)	C. I. 243	17.8	5.0	5.3	71.5	28.8	27.5	23.0	49.2	26.5
Evergreen (standard)	C. I. 583	29.7	0	.....	53.6	18.2	21.6	36.5	39.2	15.1
Kaoliang:										
Dwarf Shantung	C. I. 293	23.9	.....	.....	31.8	17.5	23.9	0	26.9	21.4
Manchu Brown	C. I. 171	48.0	16.7	19.5	48.6	18.8	23.7	12.0	38.6	36.0
Miscellaneous sorghums:										
White Yolo	K. B. 2525	0	40.9	0	0	60.3	0	1.2	35.3	0
Darso	K. B. 2536	5.3	8.8	19.9	3.0	31.8	36.3	0	21.4	27.6
White durra	C. I. 81	30.8	0	41.6	63.3	72.5	30.7	10.8	31.1	18.9
Shallu	C. I. 85	66.7	40.0	22.0	43.2	33.2	6.7	19.8	44.7	42.1
Shallu selection	K. B. 2879	.....	.....	.....	75.1	15.0	7.1	17.9	50.0	22.7
Schrock	K. B. 2541	7.4	.....	.....	12.4	1.7	25.6	0	9.2	43.5
Modoc	H. C. 2520	.....	.....	.....	58.4	29.1	43.5	14.6	28.3	11.4
Dwarf Freed	H. C. 2521	67.1	.....	.....	80.5	46.2	54.8	26.1	.....	52.3
Premo	F. C. I. 8929	19.4	28.1	0	17.7	33.3	0	5.8	16.7	0
Pierce kaferita selection	K. B. 2547	0	.....	.....	0	0	18.7	0	0	20.6
Do	K. B. 2549	52.0	0	50.6	47.2	51.8	44.2	12.8	32.9	28.4
Pierce kaferita (types 3 and 4)	K. B. 27101	15.4	.....	.....	8.3	99.0	8.3	1.6	6.6	29.5
Sudan grass	K. B. 2681	.....	.....	.....	.....	.....	.....	10.0	3.5	9.0

It will be noted that the reaction of the varieties is remarkably consistent. As sorghums hybridize very readily, off-type plants occasionally occur. Also it is exceedingly difficult at times to prevent spores of the various forms of kernel smut from getting where they are not desired. This may explain the occasional occurrence of a smutted plant in a variety known to be highly resistant, and probably is accountable for the occurrence of 1.2 per cent of form 1 (kafir smut) in White Yolo, K. B. 2525. White Yolo is regarded as immune from this smut, as has been proved in all other experiments. There is, however, the possibility that the occurrence of smut in such an instance is due to the appearance of a new form of smut. In the light of present knowledge, it is possible that the occurrence of 7 smutted feterita plants in a total of 3,638 plants grown by Reed and Melchers (7) at Manhattan, Kans., Columbia, Mo., Brooklyn, N. Y., and Rosslyn, Va., likewise may have been due to a similar phenomenon. The same holds true for the 3 smutted Standard White milo plants recorded by them in a total of 2,256 plants. The occurrence of physiologic forms of smut was not suspected by them at that time, however, and the logical explanation was an off-type plant or a susceptible hybrid.

In Table 2 are grouped the data on 23 varieties of sorghums selected from Table 1 and arranged to show their resistance or susceptibility to one or more of three forms of kernel smut. The reactions obtained indicated that the varieties used could be placed in seven definite groups. Kafir, broomcorn, kaoliang, shallu, and Red Amber sorgo are susceptible to all three forms and are designated as group 1. Group 2 consists of Dwarf Yellow milo, hegari, and White Yolo, which are susceptible to form 2 and resistant to forms 1 and 3. Some of the Red Amber×feterita hybrids and Spur feterita are resistant to all three forms and are placed in group 3. Group 4 consists of only one of the Red Amber×feterita hybrids, which is susceptible to forms 1 and 3 and resistant to form 2. Most of the feteritas and some of the kaferitas are resistant to forms 1 and 2 and susceptible to form 3, and are designated as group 5. Fargo Straightneck milo and Premo belong to group 6, being susceptible to forms 1 and 2 and resistant to form 3. Group 7 consists of two selections of the milo×kafir cross, which is resistant to form 1 and susceptible to forms 2 and 3.

In 1929 it became evident that two additional forms of kernel smut of sorghum had been found. One collection came from a feterita plant grown at the agronomy farm, Manhattan, Kans., and a second was obtained from J. H. Martin, who found it on feterita received from San Antonio, Tex., in 1927. These collections, together with the three forms previously known, were used to inoculate a limited number of sorghums, chosen as differential hosts. The results presented in Table 3 indicate that these two additional collections are distinct forms of *Sphacelotheca sorghi*. They are, therefore, designated as forms 4 and 5. Thus Dwarf Yellow milo (C. I. 332) is susceptible to form 2 and resistant to forms 1, 3, 4, and 5. White Yolo (K. B. 2525) is susceptible to forms 2 and 4 and resistant to forms 1, 3, and 5. Pierce kaferita (K. B. 2547), feterita×kafir (F. C. I. 8917), and feterita (S. P. I. 51989) are susceptible to form 3 and resistant to forms 1, 2, 4, and 5. The kafir×feterita hybrid (H. C. 2423) is susceptible to forms 3 and 5 and resistant to forms 1, 2, and 4.

TABLE 2.—*Percentage of smutted heads in sorghums inoculated with three physiologic forms of Sphacelotheca sorghi, at Manhattan, Kans., 1927 to 1929, grouped according to reaction*

Group No.	Variety, selection, or hybrid	Accession No.	Percentage of heads smutted after inoculation with—								
			Form 1 (kafir)			Form 2 (milo)			Form 3 (feterita)		
			1927	1928	1929	1927	1928	1929	1927	1928	1929
1	Pink kafir	K. B. 2506	36.7	65.8	18.8	49.1	37.2	50.0	50.3	33.3	27.8
	Acme broomcorn	C. I. 243	17.8	71.5	23.0	5.0	28.8	49.2	5.3	27.5	26.5
	Manchu Brown kaoliang	C. I. 171	48.0	48.6	12.0	16.7	18.8	38.6	19.5	23.7	36.0
	Shallu	C. I. 85	66.7	43.2	19.8	40.0	33.2	44.7	22.0	6.7	42.1
	Red Amber sorgo	K. B. 2504	26.5	32.3	17.1	10.1	19.8	17.1	----	15.1	11.6
2	Dwarf Yellow milo	K. B. 2515	0	0	0	22.8	11.3	18.0	----	0	0
	Do	C. I. 332	0	0	0	10.4	10.7	13.2	----	0	0
	Hegari	K. B. 2518	0	0	0	2.9	8.6	1.0	0	0	0
	Do	K. B. 2537	----	0	0	----	9.0	13.6	----	0	0
	White Yolo	K. B. 2525	0	0	1.2	40.9	60.3	35.3	0	0	0
3	Red Amber×feterita	K. B. 2509	0	0	0	0	0	0	0	0	0
	Do	K. B. 2562	0	0	0	0	0	0	0	0	0
	Do	K. B. 2573	0	0	0	0	0	0	0	0	0
	Spur feterita	K. B. 2540	0	0	0	0	0	0	----	0	0
4	Red Amber×feterita	K. B. 2570	21.5	25.9	13.8	0	0	0	15.9	18.1	21.3
5	Red-leafed feterita	K. B. 2543	----	----	0	----	0	0	----	4.9	3.9
	Feterita	S. P. I. 51989	0	0	0	0	0	0	24.8	17.9	2.6
	Pierce kaferita	K. B. 2547	0	0	0	----	0	0	----	18.7	20.6
	Feterita×kafir	F. C. I. 8917	----	0	0	----	0	0	----	34.4	2.4
6	Fargo Straightneck milo	C. I. 809	25.0	19.2	3.3	3.2	4.4	25.3	0	0	0
	Premo	F. C. I. 8929	19.4	17.7	5.8	28.1	33.3	16.7	0	0	0
7	Dwarf Yellow milo×Pink kafir	H. C. 257	0	0	0	----	26.1	12.8	----	12.3	5.6
	Kafir×milo 26-3-1-1	K. B. 2561	0	0	0	34.5	37.0	26.3	10.9	21.8	13.0

TABLE 3.—*Percentage of smutted heads in sorghums inoculated with five physiologic forms of Sphacelotheca sorghi, at Manhattan, Kans., 1929*

Variety, selection, or hybrid	Accession No.	Percentage of heads smutted after inoculation with—				
		Form 1 (kafir)	Form 2 (milo)	Form 3 (feterita)	Form 4 (feterita)	Form 5 (feterita)
Dwarf Yellow milo	K. B. 2515	0	18.0	0	0	0
Do	C. I. 332	0	13.2	0	0	0
White Yolo	K. B. 2525	1.2	35.3	0	12.6	0
Pierce kaferita	K. B. 2547	0	0	20.6	0	0
Feterita × kafir	F. C. I. 8917	0	0	2.4	0	0
Feterita	S. P. I. 51989	0	0	2.6	0	0
Kafir × feterita	H. C. 2423	0	*0	*18.4	0	41.6

* Percentage of smut obtained in 1928. On account of shortage of seed there are no 1929 data on the reaction of these two forms.

For aid in identifying the five physiologic forms of kernel smut (*Sphacelotheca sorghi*) the various groups of sorghums used to differentiate them are arranged in the following dichotomous key.

KEY FOR THE IDENTIFICATION OF PHYSIOLOGIC FORMS OF SPHACELOTHECA SORGHI

A. Kafir × feterita (H. C. 2423), resistant.
  B. Dwarf Yellow milo (C. I. 332), highly resistant.
    C. White Yolo (K. B. 2525), resistant ---- Form 1.
    CC. Yolo (K. B. 2525), susceptible ---- Form 4.
  BB. Dwarf Yellow milo (C. I. 332), moderately susceptible ---- Form 2.
AA. Kafir × feterita (H. C. 2423), susceptible.
  B. Pierce kaferita (K. B. 2547), feterita × kafir (F. C. I. 8917), and feterita (S. P. I. 51989), highly resistant ---- Form 5.
  BB. Pierce kaferita (K. B. 2547), feterita × kafir (F. C. I. 8917), and feterita (S. P. I. 51989), susceptible ---- Form 3.

MORPHOLOGIC CHARACTERS OF SPHACELOTHECA SORGHI

Ficke and Johnston (*1*) have shown that forms 1, 2, and 3 of *Sphacelotheca sorghi* may be separated from one another when grown on various artificial culture media, by the color, surface contour, consistency, margin, and rate of growth of the colonies. They also noted that sectoring was rather common in form 1, but rarely occurred in form 2 and was not observed at all in form 3. The cultural characteristics seemed, according to these authors, to be fairly constant. Their studies did not consider the possible morphologic differences of the fungus other than those exhibited in culture. Observations by these authors in the field did not indicate any consistent difference in shape, size, or color of the sori.

TABLE 4.—*Data on approximate length, color, and degree of rupturing of sori of five physiologic forms of Sphacelotheca sorghi in two series of smutted panicles of varieties and strains of sorghum, at Manhattan, Kans., 1929*

Physiologic form No.	Series No. of smutted panicles	Average protrusion of sori	Varieties with membranes of indicated color		Average degree of rupturing of membrane [a]
			Brown	White	
		Mm.	*Number*	*Number*	
1	1	3.7	38	0	0.9
	2	3.8	35	0	.7
2	1	4.0	20	18	2.7
	2	3.7	21	16	2.9
3	1	5.4	2	35	.7
	2	5.3	1	35	.5
4	1	3.4	39	0	.9
	2	3.8	36	0	.4
5 [b]	1	4.1	12	4	.4

[a] In recording the degree of rupturing of the membrane of the sori, the material was divided into five classes represented by the following units of measurement: 0=No rupturing; 1=very few sori ruptured and ruptured only to a slight extent; 2=few sori ruptured to a moderate extent; 3=many sori ruptured to a moderate extent; and 4=most sori greatly ruptured.

[b] Only 16 varieties were included, and 15 of the 16 panicles were only partially smutted.

In the present studies the sori of all five physiologic forms studied were carefully examined, both the chlamydospores and the membranes, as well as the general form and rupturing. Large numbers of chlamydospores of each form were measured. All measurements fell within the range given for *Sphacelotheca sorghi*, namely, 5μ to 9μ. The spores of all forms were found to be dark brown in color when observed in mass and olive brown when observed singly. Spores of all forms also were observed to be spherical or subspherical and rather thick-walled and smooth. The characteristics of the membrane of the sori were studied in some detail by choosing a typical smutted head of each of the five physiologic forms occurring on 43 different varieties or selections of sorghum and placing them on a table arranged by variety and smut form. The smutted panicles were from the same varieties as were used in the inoculation studies previously discussed. For these studies of the sori an effort was made to choose varieties that were susceptible to all five physiologic forms of the smut. Thus it was possible to compare the five forms on a number of the same varieties of sorghum. In the case of form 5, however, only 16 varieties developed smut. Observations were made on the approximate length, color, and rupturing of the sori of all five forms of smut. Both macroscopically and microscopically the sori were found to be typically

those of *S. sorghi* and did not resemble those of *S. cruenta*. After the first series of panicles had been examined and the results recorded, a second series of typical smutted heads was chosen and similarly compared. The results, as given in Table 4, are based on the two sets of observations.

The relative length of the sori of each smutted panicle was recorded as long, medium, or short. The protrusions of the sori beyond the glumes for these three classes were: Long, 5 to 7 mm.; medium, 3 to 5 mm.; short, 1 to 3 mm. Although no actual measurements were made, the range is so great, especially for the two extreme classes, that it is not likely that any error in classification occurred. In

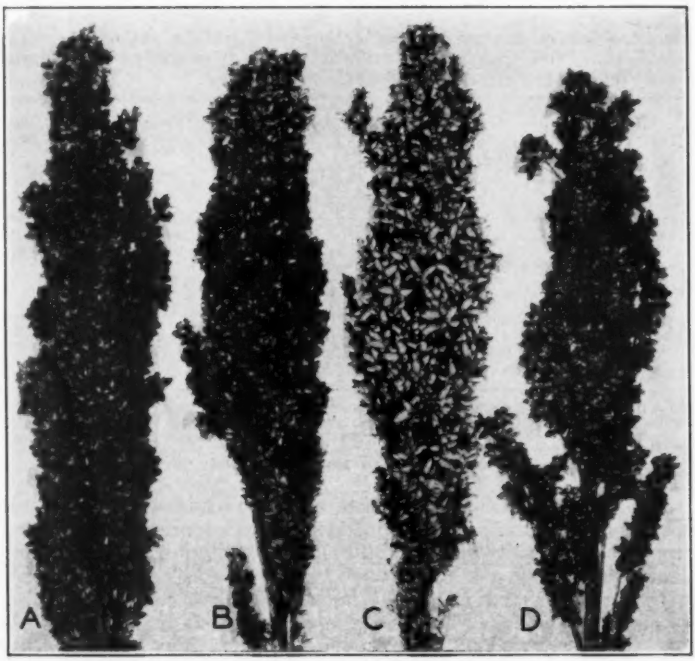

FIGURE 1.—Physiologic forms of *Sphacelotheca sorghi* on Pink kafir. A, Physiologic form 1; membranes of sori brown and mostly unruptured; sori short. B, Physiologic form 2; membranes of sori may be white or light brown, badly ruptured, spores escaping; sori moderately short. C, Physiologic form 3; membranes of sori white and mostly unruptured; sori very long. D, Physiologic form 4; membranes of sori brown and unruptured; sori moderately long

summarizing the data for Table 4, the number of individuals in each class was multiplied by the median of the class, the results added and divided by the total number of measurements. This gave what appeared to be a reasonable figure for the average length of the sori for each physiologic form. The color of the membranes of the sori was recorded as brown or white, the total number of smutted panicles showing each color for each physiologic form being given in the table.

It will be observed from the data in Table 4 that forms 1 and 4 are characterized by membranes that are brown in color and only

slightly ruptured. The sori are intermediate in length. The membranes of form 2 are either brown or white, depending on the variety, and usually they are considerably ruptured. Form 3 is characterized by long sori with white membranes that are seldom ruptured to an appreciable extent. In form 5 the sori are rather large and may have either brown or white membranes, with little rupturing. It is also characteristic of form 5 to produce only partial smutting of the panicles. Specimens of Pink kafir attacked by four different physiologic forms are shown in Figure 1, and Manchu Brown kaoliang attacked by form 5 is shown in Figure 2.

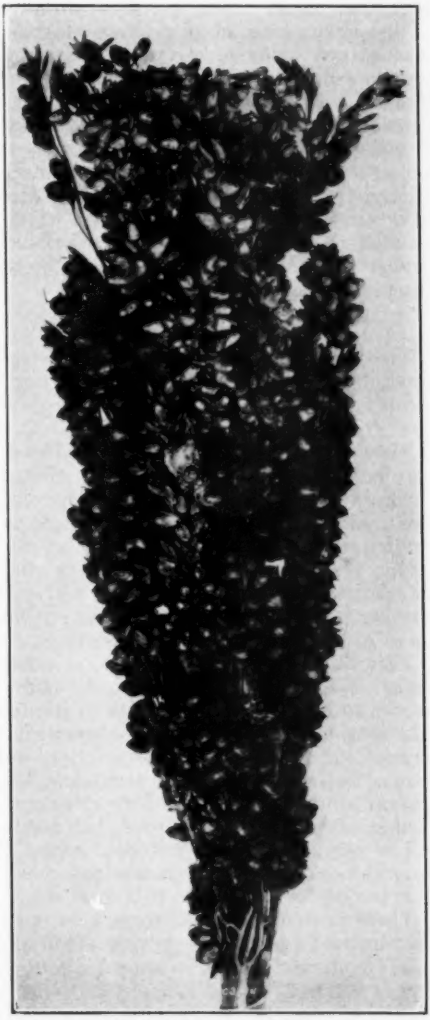

FIGURE 2.—Physiologic form 5 of *Sphacelotheca sorghi* on Manchu Brown kaoliang. A few florets in the tip of the head are smutted, while the remainder of the panicle is unsmutted. This reaction to physiologic form 5 is typical

From the data at hand it is doubtful whether the five physiologic forms of *Sphacelotheca sorghi* can be separated solely on the basis of observable differences in the sori. This has not been attempted over a series of years or under different environmental conditions. However, the data in Table 4 indicate several striking differences that may be helpful in making tentative identifications.

DISTRIBUTION AND IMPORTANCE OF PHYSIOLOGIC FORMS OF SPHACELOTHECA SORGHI

Observations and records over a period of years show that the kafir kernel smut (form 1) is most common and widespread in the United States. Form 2 is less common, but since it attacks many varieties that are susceptible to form 1 it may possibly become more common and become a problem in areas devoted to the milo crop. Such is the case in parts of New Mexico and Texas, and undoubtedly will be

true in Kansas and other States where milo is an important crop. Less is known at the present time about the performance and distribution of forms 3, 4, and 5, although the possibility of their increasing is an important consideration from the standpoint of breeding for resistance to the kernel smut of sorghum.

The occurrence of physiologic forms of kernel smut of sorghum that attack hitherto resistant or immune varieties and strains has greatly complicated the problem of breeding for resistance to kernel smut. Smut-resistant hybrids produced several years ago at the Kansas Agricultural Experiment Station are now susceptible to one or more of the forms of sorghum smut, thereby reducing the number which formerly were regarded as immune.

Seed-treatment experiments (*2*, *4*, *5*) conducted over several years have shown that sorghum kernel smut (forms 1 to 5) is effectively controlled by the copper-carbonate dust method. This method is almost exclusively used in Kansas because of its efficiency, ease of application, and cheapness.

SUMMARY

Three years' data on physiologic specialization of *Sphacelotheca sorghi* have been obtained. Eighty varieties, selections, and hybrids comprising the various groups of sorghums have been used in testing five physiologic forms.

These forms have been designated as forms 1, 2, 3, 4, and 5. They may be separated by the reactions of varieties of sorghums.

There are no outstanding morphologic differences between the chlamydospores of the five physiologic forms of *Sphacelotheca sorghi* studied.

One year's data from comparative studies of the morphologic characteristics of the sori of the five physiologic forms of *S. sorghi* on a number of sorghum varieties have shown some rather definite differences in length, color, and rupturing.

Varieties such as durra, milo, selections of feterita, darso, Dwarf hegari, White Yolo, and certain hybrids, which a few years ago were known to be highly resistant to or immune from *Sphacelotheca sorghi* infection, are now known to be somewhat susceptible to one or more physiologic forms. Of the varieties, selections, and hybrids so far grown, 1 selection of Spur feterita and 3 Red Amber × feterita crosses remain immune from all 5 forms of smut. The sorghum host range used in these studies, however, has not been exhausted.

The occurrence of physiologic forms of smut that attack hitherto resistant or immune sorghums has greatly complicated the problem of breeding for resistance to kernel smut.

There is evidence that form 1 is most common and most widely distributed in sorghum-growing areas of the United States. Form 2 is less common. Less is known about the occurrence and distribution of forms 3, 4, and 5.

Seed-treatment experiments conducted at Manhattan, Kans., have shown that forms 1 to 5 may be controlled by the copper-carbonate dust seed treatment.

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INHERITANCE OF HEIGHT IN BROOMCORN[1]

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INTRODUCTION

Broomcorn (*Holcus sorghum* L.; *Sorghum vulgare* Pers.) is a crop of which both the varietal and commercial classes are based upon differences in plant height. The varieties of broomcorn that are, or have been, produced commercially in this country are divided on the basis of height into three groups—standard (tall), western dwarf, and whisk dwarf.[2] Records show that broomcorn of the standard type was imported into the United States at an early date, while the western dwarf and whisk dwarf types appeared later, with no evidence of foreign introduction. The broomcorn dwarfs thus far observed have had shorter internodes but practically the same number of nodes as the standard type. This fact has been so apparent (figs. 1 and 2) that the evidence of detailed counts of the number of nodes is not considered necessary.

In the course of varietal improvement crosses were made between varieties of the several types of broomcorn. The unexpected occurrence of plants of standard height in a cross between two dwarf types indicated the need for a study of height inheritance in broomcorn. This paper gives the results of such an investigation.

CROSSES STUDIED

Crosses were made at the United States Dry-Land Field Station, Woodward, Okla., in 1919, between a strain of standard broomcorn (Evergreen, C. I.[3] No. 556) and a western dwarf variety (Acme, C. I. No. 243), and also between a whisk dwarf variety (Japanese Dwarf, C. I. No. 442) and the Evergreen variety. The average heights of the three parental varieties in an 8-year period were: Evergreen, 97 inches; Acme, 57 inches; Japanese Dwarf, 42 inches. The crossed seeds were planted and produced F_1 plants in 1920. In 1921 two 8-rod rows of the F_2 generation of the cross between Evergreen and Acme were grown and height data were obtained. Measurements of the parental varieties in adjoining rows also were obtained.

A number of broomcorn crosses were again made in 1923 to study the inheritance of the broomcorn height factors. These included Acme × Evergreen, Japanese Dwarf × Acme, and Japanese Dwarf × Evergreen. It was expected that height data could be obtained from the F_2 generations of these crosses in 1925. The season, however, was unfavorable for normal height development of most broomcorns, especially the standard varieties, and consequently height measurements were not taken.

In 1927 to obtain additional data a cross was again made between the Acme and Japanese Dwarf varieties.

[1] Received for publication June 30, 1931; issued February, 1932.

[2] SIEGLINGER, J. B. BROOMCORN EXPERIMENTS AT THE UNITED STATES DRY-LAND FIELD STATION, WOODWARD, OKLA. U. S. Dept. Agr. Tech. Bul. 51, 32 p., illus. 1928.

[3] C. I. refers to accession number of Division of Cereal Crops and Diseases, formerly Office of Cereal Investigations.

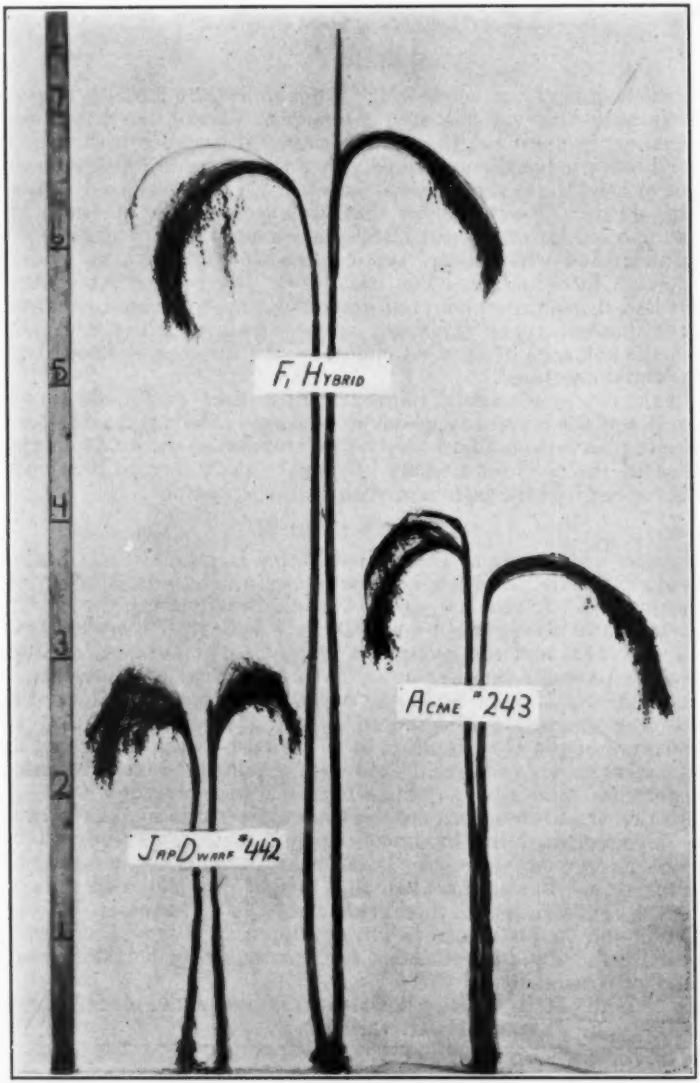

FIGURE 1.—Plants of the F_1 cross Acme × Japanese Dwarf broomcorn, and the parents

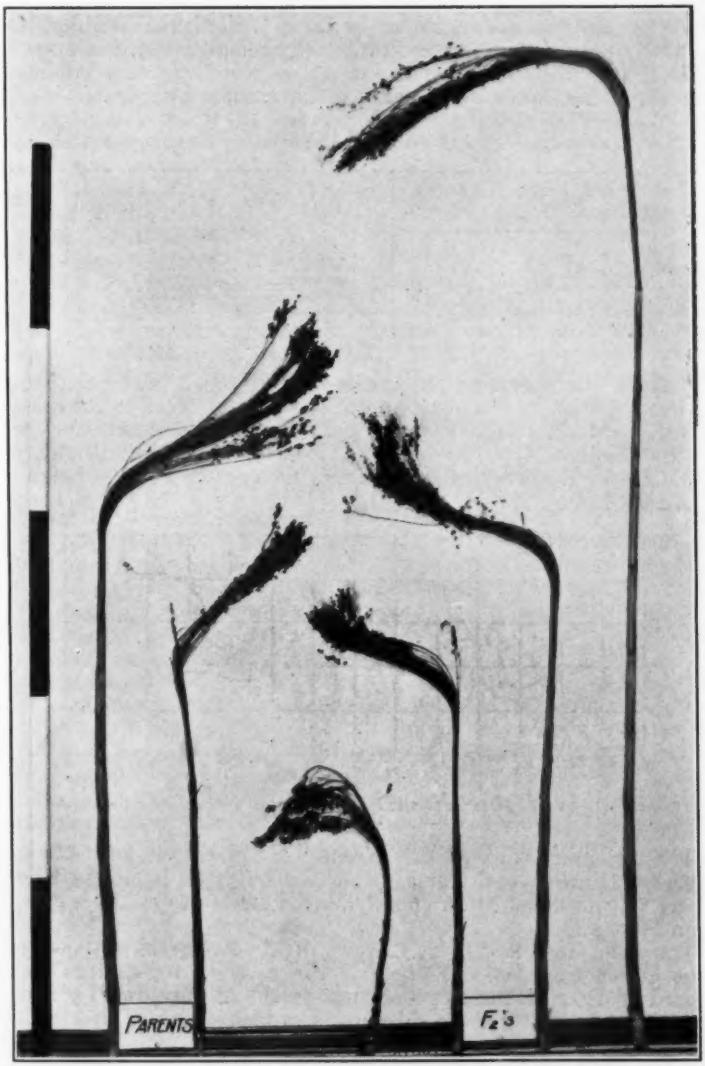

FIGURE 2.—Stalks representative of the four phenotypes of the F_2 cross, Acme × Japanese Dwarf broomcorn. (Parent plants at left)

RESULTS

STANDARD × DWARF CROSSES

The average heights of the F_1 plants in comparison with the dwarf and standard parents are shown in Table 1. It is realized that these numbers are too small to be statistically reliable, but it is apparent that the F_1 plants were about as tall as the Evergreen (standard) parent. This shows a dominance of the standard height.

TABLE 1.—*Height of parents and F_1 broomcorn crosses in 1924*

Variety	Group	Number of plants	Height
			Inches
Evergreen	Standard	3	103 ±0.4
Acme	Western dwarf	4	62 ± .6
Japanese Dwarf	Whisk dwarf	6	41.8± .8
Japanese Dwarf × Evergreen		1	99
Acme × Evergreen		4	101.5±1.6

The results from the F_2 progenies in 1921 (fig. 3) showed a simple 3:1 segregation of standard and western dwarf types in the cross of Evergreen × Acme and its reciprocal. Of the 767 plants, 582 were of the standard type, averaging 112.0 ± 0.20 inches in height, and 185 were of the western dwarf type, having an average height of 60.5 ± 0.3 inches.

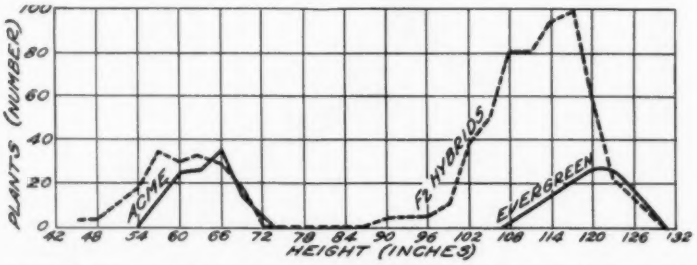

FIGURE 3.—Height of F_2 plants of a broomcorn cross, Evergreen × Acme, and of the parent varieties, 1921

The adjoining rows of the parental Evergreen variety averaged 119.6 ± 0.3 inches, and Acme averaged 63.8 ± 0.26 inches in height. There was no difficulty in classifying the two F_2 types, as is evident from Figure 3.

The following year, 1922, 12 rows of the Evergreen × Acme cross were grown from selfed F_2 heads. Six of these rows, from plants classed as dwarf in the F_2 generation, produced only dwarf plants in the F_3 generation. Of the six rows from plants classed as standard in the F_2 generation, five segregated for standard and dwarf plants and one was pure for standard height.

The Acme × Evergreen cross in 1925 again produced standard and western dwarf types in the F_2 generation in the ratio of 3:1, thus confirming the 1921 results on this cross.

The above results show that the standard (tall) type of broomcorn differs from the western dwarf type by a single height factor.

WHISK DWARF × WESTERN DWARF CROSSES

The average height, in 1924, of 12 F_1 plants of a cross between Japanese Dwarf (whisk dwarf) and Acme (western dwarf) broomcorn was 101.4 ± 0.8 inches. In this experiment six Japanese Dwarf plants averaged 41.8 ± 0.8 inches and four Acme plants averaged 62 ± 0.6 inches in height. Plants of the F_1 hybrid and the two parents are shown in Figure 1. Evergreen (standard) plants under the same conditions averaged 103 ± 0.4 inches in height, or almost the same as the F_1 plants from the cross between the two dwarf varieties. This indicates the presence of complementary height factors in the two dwarf types.

The F_1 plants of the above cross grown in 1928 averaged 86 inches in height, as compared with 53 inches for Acme and 40 inches for the Japanese Dwarf parent.

The Japanese Dwarf × Acme cross produced plants in the F_2 generation, in 1925, corresponding in height to the F_1 (standard) plant and to each of the dwarf plants. In addition there appeared a fourth class of extremely short or "double dwarf" plants. (Fig. 2.) The plants of the dwarf groups could not all be accurately classified for height, however, because of the unfavorable growing conditions in 1925.

The double dwarf type of plant is not represented by any commercial variety of broomcorn. Extra dwarf or double dwarf plants have been observed, however, in other sorghum groups, particularly in milo, of which the Double Dwarf variety is extensively grown. The double dwarf type of broomcorn was first observed in 1921 in the F_2 progeny of a natural hybrid plant of standard height found in a field of Japanese Dwarf broomcorn. The 275 plants of this natural hybrid population segregated into 167 standard plants, 47 western dwarf, 48 whisk dwarf, and 13 double dwarf plants, closely approaching a dihybrid ratio. A true breeding strain of double dwarf broomcorn from this cross has been maintained since 1921.

Remnants of seed from F_1 plants of the Japanese Dwarf × Acme cross were sown in 1926, but they germinated poorly. Five 8-rod rows contained a total of only 196 plants. These were measured and classified, and observed groupings are compared in Table 2 with the calculated numbers based on a 9:3:3:1 ratio. This distribution gives a χ^2 value of 1.97, with $P = 0.579$, which indicates that a deviation as great as or greater than that observed may be expected to occur about six times in 10 as a result of random variation.

TABLE 2.—*Broomcorn plants from F_1 seed measured and classified, with calculated numbers based on a 9:3:3:1 ratio*

Phenotype	Observed	Calculated	Mean height
			Inches
Standard	115	110.25	95.8
Western dwarf	40	36.75	57.2
Whisk dwarf	32	36.75	44.4
Double dwarf	9	12.25	24.9

In 1929 five 8-rod rows of the F_2 generation produced 525 plants. These and a number of plants of the parent varieties were measured for height. Although the development of the F_2 plants was fairly

satisfactory, there was not a clear-cut difference between the three dwarf classes in 1929 but a partial blending from one height class to the next. (Fig. 4.) There was no overlapping between the height of western dwarf and that of standard, though the difference in height was less pronounced than it would have been had the plants been grown under better environmental conditions. The measurements of the parent and F_2 populations and the segregation of the latter are shown in Table 3 and Figure 4.

TABLE 3.—*Height of parents and F_2 plants of a cross between whisk dwarf and western dwarf broomcorn in 1929*

Height class	Total plants	Range in height	Mean height	Standard deviation	Coefficient of variability
Parents:	*Number*	*Inches*	*Inches*		
Whisk dwarf	45	32–45	39.0±0.3	3.22	8.25
Western dwarf	53	49–64	57.6± .3	3.20	5.56
F_2 hybrids:					
Double dwarf	34	13–31	24.6± .5	4.58	18.62
Whisk dwarf	94	32–47	40.5± .28	4.08	10.07
Western dwarf	104	48–68	55.6± .29	4.44	7.99
Standard	293	73–112	91.4± .29	7.39	8.08

About 200 of the F_2 plants were selfed, and in 1930 progenies from 112 typical plants, representing all height classes, were grown in head

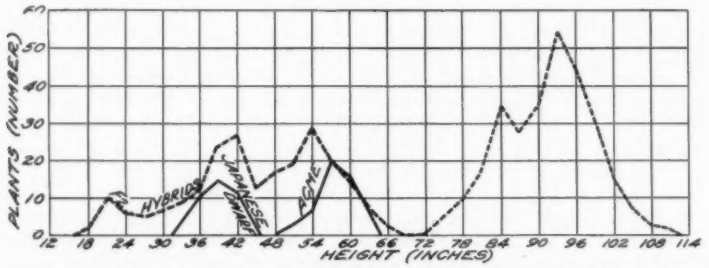

FIGURE 4.—Height of F_2 plants of a broomcorn cross (Acme × Japanese Dwarf) and of the parent varieties, 1929

rows to observe their genotypic behavior in the F_3 generation. The study of these F_3 progenies showed that the classification of the plants for standard height in the F_2 generation was correct. All of the 44 standard F_2 plants produced some standard plants in the F_3 generation. There was a discrepancy in classifying the double dwarfs because several of the very short plants produced heterozygous whisk dwarf progenies in the F_3 generation. Also, a few F_2 plants that were classed as western dwarf proved to be whisk dwarf. Based on the proportions observed and the behavior of the 112 F_3 progenies produced in 1930, the corrected numbers in the entire F_2 population of 525 plants were as shown in Table 4.

This distribution gives a χ^2 value of 6.11 and a P value of 0.108. A fit as poor as the foregoing may be expected once in nine or ten times as a result of random variation.

TABLE 4.—*Observed and calculated numbers from entire F_2 population of 525 broomcorn plants*

Phenotype	Observed number	Calculated number	Deviation
Double dwarf	22	32.8	10.8
Whisk dwarf	114	98.4	15.6
Western dwarf	96	98.4	2.4
Standard	293	295.4	2.4

Of the 44 F_3 progenies from standard phenotypes in F_2, 3 were pure standard, 6 segregated into approximately 3 standards to 1 whisk dwarf, 18 segregated into standards and western dwarfs, and 17 produced standard, western dwarf, whisk dwarf, and double dwarf plants. There was no noticeable relation between the height of the F_2 standard plants and their genotypic constitution, as determined by growing F_3 generations from them.

DISCUSSION

From the above results it is apparent that both western dwarf (Acme) and whisk dwarf (Japanese Dwarf) types of broomcorn differ from standard (Evergreen) broomcorn by single height factors. The height factor present in western dwarf is not the same as the one in whisk dwarf broomcorn. For convenience the height factors concerned may be considered as *AAdd* in western dwarf, *aaDD* in whisk dwarf, and *AADD* in the standard type. The F_1 cross of the two dwarfs gives a plant of the constitution *AaDd*. The factors *A* and *D* are completely dominant, as the F_1 plant *AaDd* is as tall as a plant of *AADD* constitution. In the F_2 generation the following factorial combinations are obtained:

4 *AaDd*	2 *Aadd*	2 *aaDd*	1 *aadd*
			1 double dwarf
2 *AaDD*	1 *AAdd*	1 *aaDD*	
	3 western dwarf	3 whisk dwarf	
2 *AADd*			
1 *AADD*			
9 standard			

Examples of each of the above genotypes were obtained in the 112 strains in the F_3 generation in 1930.

Because of the lack of information regarding the origin or introduction of the whisk dwarf and western dwarf types, it is probable that both originated as mutations from some standard variety by the loss of a single but different height factor. The whisk dwarf appeared on farms about 1860, and the western dwarf type some 20 years later.

The dwarf broomcorns have a parallel in corn dwarfs,[4,5] with the exception that broomcorn reproduces normally even in the double recessive condition. Also, there is no apparent reduction in the quantity of seed produced by the presence of either recessive dwarf factor in broomcorn. (Fig. 2.) Based on the corn analogy,[6] standard broomcorn would be considered as the normal type.

[4] EMERSON, R. A., and EMERSON, S. H. GENETIC INTERRELATIONS OF TWO ANDROMONOECIOUS TYPES OF MAIZE, DWARF AND ANTHER EAR. Genetics 7: 203–236, illus. 1922.
[5] KEMPTON, J. H. INHERITANCE OF DWARFING IN MAIZE. Jour. Agr. Research 25: 297–321, illus. 1923.
[6] EMERSON, R. A., and EMERSON, S. H. Op. cit. (See footnote 4.)

Standard broomcorn attains a height of from 9 to 12 feet under favorable conditions. The F_1 plants of crosses between dwarf broomcorn and any other sorghum are about this same height, and thus suggest a simple explanation for at least a part of the "hybrid vigor" obtained in the F_1 crosses. Other sorghums apparently usually carry the missing *D* or other complementary height factors, the result in crossing dwarf broomcorn with other sorghums being an F_1 plant the height of standard broomcorn and taller than most of the common sorghums. The F_1 plants of crosses between different sorghum groups usually are tall and exhibit hybrid vigor. The two height factors here described, however, do not account for other manifestations of hybrid vigor in sorghums, such as late maturity, thick culms, and an increased number of nodes.

SUMMARY

Broomcorn is divided into varietal and commercial classes mainly on the basis of relative height.

In crossing a standard (tall) broomcorn with western dwarf or whisk dwarf broomcorn, an F_1 plant the height of the standard parent is obtained. A single-factor segregation of three tall to one dwarf is obtained in the F_2 generation.

A cross of western dwarf and whisk dwarf broomcorns gave an F_1 plant the height of standard broomcorn. The F_2 generation gave a 2-factor segregation of 9 standard to 3 western dwarf to 3 whisk dwarf to 1 double dwarf.

Considering standard broomcorn as possessing two height factors, *A* and *D*, the western dwarf lacking the *D* factor, and whisk dwarf lacking the *A* factor, a simple explanation is apparent for the standard height of the F_1 cross between these two dwarfs and for the tall F_1 plants obtained when either type of dwarf broomcorn is crossed with other sorghums. The tall F_1 plants usually are considered entirely a result of hybrid vigor.

DEHISCENCE OF THE BOLL OF LINUM RIGIDUM AND RELATED SPECIES [1]

By A. C. DILLMAN, *Associate Agronomist*, and J. C. BRINSMADE, JR., *Assistant Agronomist, Division of Cereal Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture* [2]

INTRODUCTION

The purpose of this paper is to describe the mechanism of a very unusual method of dehiscence of the boll or capsule in the yellow-flowered flax, *Linum rigidum* Pursh, and in several related species native to the Great Plains area of North America extending from Canada to Mexico. The dehiscence of *L. rigidum* is entirely different from that of other species of the genus Linum and of dehiscent fruits of other plants.

In the common cultivated flax, *Linum usitatissimum* L., the bolls are either semidehiscent, that is, they open at the apex and crack slightly along the margins of the segments, as in the fiber and seed flaxes commonly grown in Europe and the United States, or they are indehiscent, as in most varieties of Argentina and India.[3] In another distinct type, *L. usitatissimum crepitans* Bonningh., the bolls are widely dehiscent so that the seeds fall as soon as the bolls are ripe. Several regional strains of this dehiscent flax have been described recently by Elladi,[4] a Russian investigator. This variety is grown to a limited extent in the Ukraine and is found also in Portugal, Spain, Austria, Germany, and eastern Russia. Dehiscence occurs also in *L. angustifolium* Huds., which Tammes [5] suggests may be the wild prototype of our cultivated flax, as the two species hybridize readily and produce fully fertile seeds. Prompt dehiscence of the ripe bolls also occurs in most wild species, including *L. perenne* L. of Europe and *L. lewisii* Pursh, the somewhat similar perennial flax, which is native to the Great Plains and Rocky Mountain region of North America.

In the several species mentioned, dehiscence is due simply to dehydration and shrinkage of certain tissues, or to the greater or unequal shrinkage of certain parts of the boll. The semidehiscent bolls of common flax, and even the fully dehiscent bolls of the variety *crepitans*, will close if wet by rain or dew, and open again as they dry out.

DEHISCENCE IN LINUM RIGIDUM

In the species *Linum rigidum* and the several evidently related species the mechanism of dehiscence, if it properly can be called dehiscence, is very different from the species described above. In *L. rigi-*

[1] Received for publication June 30, 1931; issued February, 1932.

[2] The writers desire to acknowledge the generous cooperation of F. H. Hillman, botanist, Division of Seed Investigations, Bureau of Plant Industry, who made the drawings shown in fig. 2, and of Merritt N. Pope, agronomist, Division of Cereal Crops and Diseases, who made the section of the boll of *Linum rigidum* shown in fig. 3.

[3] DILLMAN, A. C. DEHISCENCE OF THE FLAX BOLL. Jour. Amer. Soc. Agron. 21: 832-833, illus. 1929.

[4] ELLADI, E. V. FLAX WITH DEHISCENT CAPSULES. Trudy Prikl. Bot. i Selek. (Bul. Appl. Bot. and Plant Breeding) 22: 455-471, illus. 1929. [In Russian. English summary by C. Elladi, p. 470-471.]

[5] TAMMES, T. THE GENETICS OF THE GENUS LINUM. Bibliographia Genetica 4: 1-36. 1928.

dum the ripe bolls remain tightly closed however dry they may become, but open wide when wet by rain or dew. This mechanism has not been described heretofore, so far as the writers have been able to learn. It was first observed in July, 1926, at Mandan, N. Dak., where the species is widely distributed on the native short-grass sod. In examining some plants one morning when they were wet with dew it was seen that the bolls were wide open, whereas they had been closed the previous evening. Later in the day some dry plants were dipped in water and the bolls opened at once.

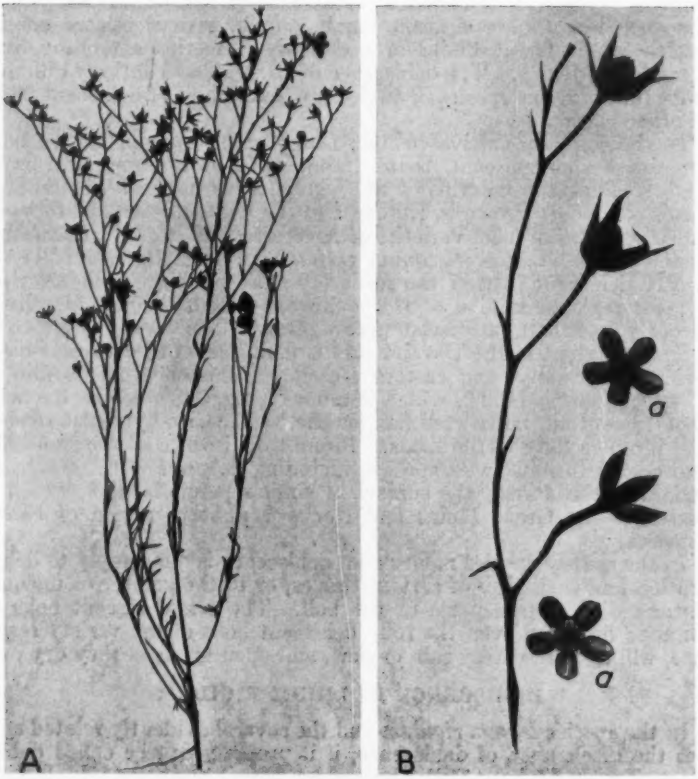

FIGURE 1.—A, Plant of *Linum berlandieri* showing the mature bolls in dry condition. B, A panicle branch and detached bolls (*a*), which were dipped in water just before the photograph was taken, showing the appearance of the bolls as they open when wet. Plants collected near San Antonio, Tex., May 24, 1931

A photograph of a plant of *Linum berlandieri* Hook., a species very similar to *L. rigidum*, collected near San Antonio, Tex., is shown in Figure 1. The appearance of the mature bolls as they open when wet can be seen on the panicle branch (B) and in the single bolls (*a*), which were dipped in water just before the photograph was taken. The immature bolls, with sepals still attached, did not open.

THE ORGAN OF DEHISCENCE

The organ of dehiscence in *Linum rigidum* is shown in Figure 2. The ovary of the flax flower consists of five carpels which form the five segments of the mature boll or capsule. In *L. rigidum* the five carpels separate in the mature boll (fig. 2, A, *a*), each carpel having two seeds inclosed in the membranous sac (endocarp), which has openings at the apex through which the seeds may escape. (Fig. 2, C, *a*.) The two seeds in each carpel are separated by a septum attached to the outer wall. Each segment of the boll is attached to the receptacle by a hingelike organ which consists of two distinct tissues. The outer tissue, which functions as a hinge, is wax yellow, translucent, tough, pliable, and nonabsorbent. (Fig. 2 A, *b;* B, *b*.) The inner tissue consists of four or five rows of colorless hygroscopic motor cells which absorb free water very rapidly. (Fig. 2, C, *b*.) If a small drop of water is placed on the outer tissue at the point *b* it

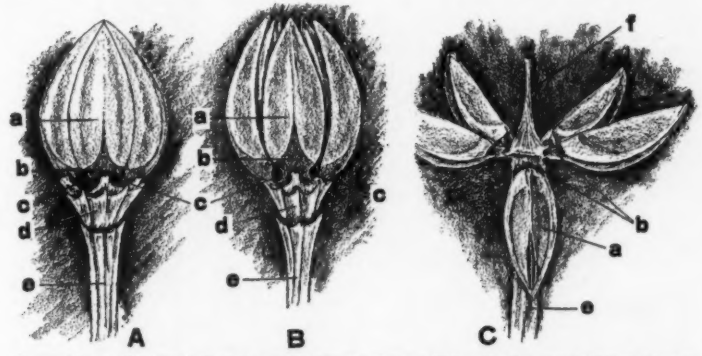

FIGURE 2.—A, Mature boll of *Linum rigidum* in dry condition; B, the same boll beginning to open a few seconds after a drop of water had been placed at the orifice *c;* C, the boll fully open. The five segments (*a*) of the boll are separated and each is attached at its base to the receptacle by a hingelike organ, *b*. The minute orifice (*c*) allows water to enter the boll, where it is rapidly absorbed by the colorless tissue C, *b*, which swells and pushes outward the separate segments, thus opening the boll. The fluted pedicel (*e*) bears a sort of capital (*d*), which in overripe plants is finally separated by abscission from the remainder of the pedicel. The growing seeds (ovules) appear to be attached by a short placental thread to the apex of the central placental column shown in C, *b*

will not be absorbed. If, however, the drop of water is moved to the margin of the orifice (fig. 2, A, *c*), it will be absorbed rapidly by the inner tissue of the organ and in about 20 seconds the boll will begin to open. (Fig. 2, B.) In about a minute the boll will be completely open as in Figure 2, C.

This mechanism suggests the mechanical principle of the thermograph in which the differential expansion of two metals due to temperature changes is made use of. In this organ of the flax boll the differential expansion, due to absorption of water by the inner tissue and nonabsorption by the waxlike outer tissue, pushes outward the separate segments, thus opening the boll. A small drop of water, or a film of water in the form of dew, is sufficient to open the boll. The boll will open completely in about 1 minute after wetting, and will close again as soon as the inner tissue dries out, usually in 5 to 10 minutes in a dry warm room.

This remarkable organ is not injured by use or incapacitated by reasonable age. The bolls of one plant in the possession of the senior writer have opened and closed probably a hundred times by wetting and drying during a period of two years. The bolls of specimens 20 to 50 years old in the National Herbarium at Washington opened readily when a drop of water was placed on them. A specimen of *Linum multicaule* Hook. collected in 1846 (now 85 years old) reacted weakly to warm water.

Photomicrographs of a section of the boll of *Linum rigidum* are shown in Figure 3. In A, the section shows one segment of the boll with the inclosed seed (*a*), the motor organs of two segments (*b*), the

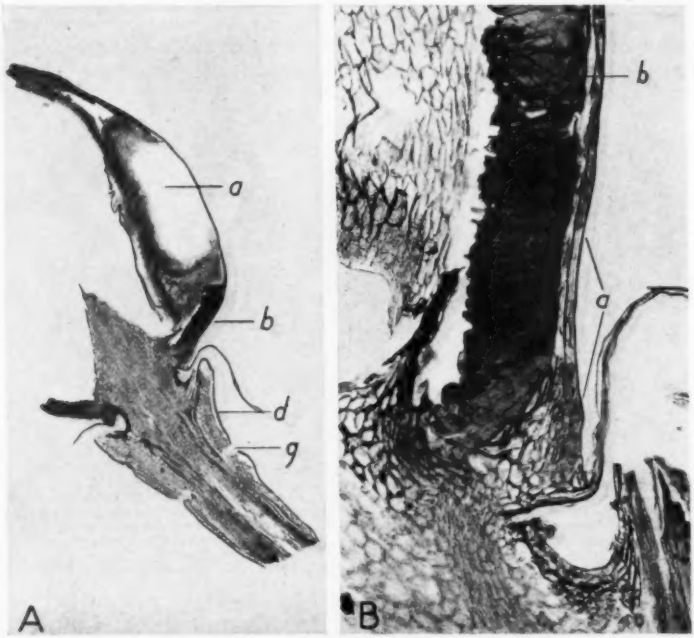

FIGURE 3.—A, Vertical section of the boll and pedicel of *Linum rigidum*, showing outline of a seed, *a*; the motor organ, *b*; the upper capitallike portion of the pedicel, *d*; and the point of abscission, *g*. × 12. B, Motor organ, shown in detail. The lignified cells, *a*, form a straplike hinge to which the mass of water-absorptive motor cells, *b*, are attached. × 72

capitallike portion of the pedicel (*d*), and the area of abscission (*g*). It is apparent that abscission is not due to the formation of special cork cells, as occurs in many plants, but is simply the separation of certain cells in a definite area of the cortex of the pedicel. In B, the motor organ is shown in more detail. It consists of a supporting tissue (*a*) of thick-walled cells which form the straplike hinge, and a mass (*b*) of colorless thin-walled motor cells. By the use of a microscope these motor cells can be seen to expand instantly when a film of water is added to a thin dry section of the organ. The section shown in this figure was stained with two dyes, the motor

cells staining dark violet with haematoxylin and the straplike tissue staining red with safranine.

SHEDDING OF THE BOLL

This mechanism of dehiscence appears to be a remarkable provision of nature to assure the distribution of the seed on wet soil under conditions favorable for germination. In practice, however, it is not highly effective. The seeds may be retained for a considerable time within the sacs, but finally emerge through the openings at the tip of the segments. (Fig. 2, C, *a'*.) The mere opening of the boll after wetting is not sufficient to insure shedding of the seeds. The application of some force, as a beating rain or a high wind, seems to be required to cause the seed to be shed. Moreover, the seeds are slow to germinate; a considerable period of wet weather, or more likely a freezing temperature, is required before the seeds will grow. As a matter of fact, the bolls may be shed from the plant before the seeds escape.

The shedding of the boll is brought about by abscission tissue which cuts off the upper portion of the short pedicel in the form of a sort of capital (fig. 2, A, *d*), to which the boll is attached. The fluted pedicel (*e*) with its capital is suggestive of a minature Corinthian column. In overripe plants this part (*d*) of the pedicel separates at the line of abscission (the dark line shown in the drawing) from the remainder of the pedicel. The bolls also are sometimes broken off at the receptacle, the place of attachment to the pedicel, instead of at the usual line of abscission.

SPECIES OF LINUM DEHISCENT WHEN WET

In the classification of the family Linaceae, Small [6] in his key to the genus Cathartolinum distinguishes a group of species as follows: "Sepals deciduous; capsules with cartilaginous thickenings at the base. VII. Rigida." No further mention is made of these "cartilaginous thickenings" in the detailed descriptions of the species listed. This is the only reference that the writers have found to this organ of dehiscence in *Linum rigidum* and related species.

The absence of sepals is characteristic of the ripe bolls. The deciduous sepals are broken off by the first opening of the mature bolls, which occurs when they are wetted by dew or rain. Sepals are present on the green and immature bolls.

The senior writer has examined all species of Linum and Cathartolinum found in the National Herbarium and has observed that this organ of dehiscence is present only in the species listed by Small in his groups Rigida and Multicaulia. In a few immature specimens it could not be determined whether the mechanism was present, although very likely it is characteristic of all species in the group Rigida. It was definitely present and operative, when a drop of water was applied to the bolls, in one or more specimens of the species listed in Table 1.

[6] SMALL, J. K. LINACEAE. *In* North American Flora 25 (pt. 1): 67–87. New York. 1907.

TABLE 1.—*Species of Cathartolinum in the National Herbarium in which the bolls were found to be dehiscent when wet*

National Herbarium specimen No.	Species	Locality	Year collected
691099	Cathartolinum puberulum	New Mexico	1912
891067	do	do	1916
13717	C. vernale	Mexico boundary survey	
739639	do	New Mexico	1902
	C. alatum		
359825	C. australe	Chihuahua, Mexico	1899
13713	C. rigidum	New Mexico	1869
265733	do	Kansas	1893
13709	do	Oklahoma	1891
504824	C. berlandieri	Texas	1914
1004457	C. aristatum	Puebla, Mexico	1907
13691	C. multicaule	Texas	1846
569482	do	Oklahoma	1913

As stated above, the organ was not found in any species except in Group VII, Rigida, and the one species *Cathartolinum multicaule* in Group VIII, Multicaulia. It is probable that this species properly belongs in the group Rigida.

This organ of dehiscence is so distinctive that it might very well be used as a generic character to distinguish the group of species in which it is present. It is very evident that these species are closely related, and, so far as known, they are native to the Great Plains and adjacent areas in North America. The character of dehiscence in these species is just opposite to that of other species of Linum and of the dehiscent pods of other plants. The bolls of these species open by wetting, the others by drying. Possibly this distinctive organ and its operation should be defined by new terms.

In Britton's Manual [7] *Linum rigidum* is described as "perennial (?)." At Mandan, N. Dak., *L. rigidum* is either an annual or a winter annual. Numerous mature plants have been marked from time to time, but none of them has lived over winter. Seeds have been sown in the fall, but germination has not been observed except in the fall of 1930, when a long period of wet weather brought about germination. It is probable that many plants are winter annuals, the seedlings overwintering under the protection of prairie grass and snow.

SUMMARY

This paper describes a peculiar plant organ that occurs in the bolls or capsules of the yellow-flowered flax, *Linum rigidum* Pursh, and in those of several related species native to the Great Plains of North America. The capsules of these species open when wet by rain, in contrast to those of other flaxes and the pods of legumes, which dehisce by drying. The opening of the capsule in *L. rigidum* is dependent on the definite action of a hingelike organ which forms the attachment of each segment of the capsule to the receptacle. The five segments of the boll are pushed open by the rapid expansion of the inner tissues of the organ, which absorb water through minute orifices at the base of and between the segments. So far as the

[7] BRITTON, N. L. MANUAL OF THE FLORA OF THE NORTHERN STATES AND CANADA. 1080 p. New York. 1901.

writers are aware, this organ of plant movement has not heretofore been described.

It is suggested that new terms be used to distinguish this organ and its action from the dehiscence of dry capsules. It is believed, also, that a separate genus might properly be made of the group of related species now included in the genus Cathartolinum of Small, which are distinguished by this peculiar organ of the capsule. The organ probably occurs in all of the 14 species listed by Small under his groups Rigida and Multicaulia.

EFFECT OF TEMPERATURE ON RATE OF DECAY OF SUGAR BEETS BY STRAINS OF PHOMA BETAE [1]

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INTRODUCTION

Tompkins and Nuckols [4] noted recently the importance of *Phoma betae* (Oud.) Frank as a wound parasite of stored sugar beets. No scientific data are available concerning the effect of environmental conditions that might conceivably favor or retard penetration of the exposed tissues of the sugar-beet crown by the fungus. During the years 1926 to 1929 the writers made detailed ecological observations at commercial storage piles in northern Utah. Accumulated evidence soon indicated that temperature probably constitutes one of the most important of the environmental factors associated with crown rot of the sugar beet in storage.

This paper reports the results of studies on the relative rate of decay of sugar beets brought about by four isolations of *Phoma betae* under controlled temperature conditions and on the relation of temperature and storage period to the quantitative effects of each of these four isolations as decay producers.

STRAINS OF PHOMA BETAE USED

Four isolations of *Phoma betae* were employed in the experiments herein reported. The sources of this material are shown in Table 1. All the pure cultures were originally obtained by making monosporous cultures, following which Brown's method [5] was used in order to insure freedom from bacterial contamination. The isolations were then thoroughly tested for pathogenicity to mature beets by inoculating freshly exposed crown tissues.

TABLE 1.—*Source of material of Phoma betae used for inoculation*

Culture No.	Source (Logan, Utah)	Date of original isolation
252	Field	Oct. 18, 1927
259	Storage pile	Nov. 30, 1927
260	do	Do.
261	do	Do.

[1] Received for publication July 14, 1931; issued February, 1932.
[2] Resigned Jan. 15, 1930.
[3] Resigned Nov. 30, 1929.
[4] TOMPKINS, C. M., and NUCKOLS, S. B. DEVELOPMENT OF STORAGE DISEASES IN SUGAR BEETS RESULTING FROM HOOK INJURY. Phytopathology 18: 939–941, illus. 1928.
——— and NUCKOLS, S. B. THE RELATION OF TYPE OF TOPPING TO STORAGE LOSSES IN SUGAR BEETS. Phytopathology 20: 621–635, illus. 1930.
[5] BROWN, W. TWO MYCOLOGICAL METHODS. I. A SIMPLE METHOD OF FREEING FUNGAL CULTURES FROM BACTERIA. II. A METHOD OF ISOLATING SINGLE STRAINS OF FUNGI BY CUTTING OUT A HYPHAL TIP. Ann. Bot. [London] 38: 401–404. 1924.

The size of spores was determined only for isolations 252 and 259, since isolations 260 and 261 did not readily form mature pycnidia on prune agar except with extreme age. Pycnidia selected from 20-day-old cultures were crushed in glycerin. One hundred spores from each of the two isolations were measured with a filar micrometer. The mean length and the mean width of the spores from both isolations are given in Table 2.

TABLE 2.—*Measurements of spores from two 20-day-old cultures of Phoma betae*

Strain No.	Length	Width
	μ	μ
252	5.595±0.047	3.815±0.028
259	4.756± .073	3.349± .036
Difference	.839± .087	.466± .046

The spores of isolation 252 are significantly longer and wider than those of isolation 259, as shown by the differences 0.839 ± 0.087 and 0.466 ± 0.046, respectively. Because of (1) this significant difference in spore size, (2) distinct differences between the various isolations observed in culture, and (3) the difference in aggressiveness shown in attacking sugar-beet tissue, discussed later in the paper, these four isolations are considered as representing four strains of *Phoma betae*.

METHODS USED IN DETERMINING RATE OF DECAY

Since the technic used in the experiments varied, details are given for each experiment in chronological order. In all experiments sound, healthy beets were used. In order to expose fresh tissue free from cork cells all crown tissues above the base of the lowest leaf scar were removed prior to inoculation by means of a steel knife that had been dipped in 95 per cent alcohol and flamed. Either whole beets or sections of beets were inoculated as indicated below. The inoculum, comprising vigorous growing colonies of the four strains of the fungus, was prepared in Petri dishes containing approximately 20 c. c. of freshly prepared prune agar. The age of the inoculum varied slightly in each of the experiments. The inoculum with agar substratum was added in each case so as to cover the exposed surface of the crown. The inoculum was thus in direct contact with the cut surface of the beet tissue, and the agar served as a protective agency against desiccation. As a further precaution against premature drying of the inoculum, a piece of sterile absorbent cotton moistened with sterile water was placed on top of the inoculum and held there by means of adhesive tape. Unless otherwise indicated, inoculated beets were rolled up in glassine bags to prevent drying of the host and fungus.

Details for each of the four experiments are as follows:

Experiment 1

Storage period, January 10 to February 20, 1929, 41 days.
Age of inoculum, 15 days.
Purpose of experiment:
(1) To determine the effect of temperature on the organism and the host.
(2) To determine rate of decay production for each fungus strain.

Beets, after inoculation with the various strains, were placed at the four different temperatures in the control chambers, as shown in Table 3.

At the completion of the storage period, the weights of the whole beet, of diseased tissue, and of the remaining healthy tissue were taken.

Experiment 2

Storage period, February 20 to April 1, 1929, 40 days.
Age of inoculum, 17 days.
Purpose of experiment: Same as in experiment 1.
In order to avoid as a possible source of error the variability in morphology and chemical composition of individual beets, one-fourth sections of beets were used instead of whole beets. The crown tissues were removed as in experiment 1, and the beets were then quartered longitudinally.
Procedure for determining the effect of (1) temperature and (2) pathogenicity of strains:

(1) The four one-fourth sections of the same individual beet were inoculated with the same fungus strain, after which each one of these quarters was subjected to a different temperature in order to determine the effect of temperature on the fungus strain and the host.

(2) In a second test each one of the four sections of an individual beet was inoculated with a different strain of the fungus and subjected to similar controlled storage conditions in order to determine more accurately the relative pathogenicity of each organism.

At the conclusion of the experiment observations were made on the weight of both diseased and healthy tissue.

Experiment 3

Storage period, April 4 to May 8, 1929, 34 days.
Age of inoculum, 20 days.
Purpose of experiment: Same as in experiments 1 and 2 and, in addition, to test methods of procedure with primary reference to moisture exchange.
The two procedures were used as indicated in experiment 2.
The inoculated beet sections were rolled in glassine bags.
The data included the weight of the beet sections before and after storage and the weight of the diseased tissue.

Experiment 4

Storage periods:
November 26 to December 8, 1929, 12 days.
November 26 to December 20, 1929, 24 days.
November 26, 1929, to January 2, 1930, 37 days.
November 26, 1929, to January 13, 1930, 48 days.
Age of inoculum, 20 days.
Purpose of experiment: Same as in experiments 1, 2, and 3, but conducted under better controlled conditions of moisture.
Instead of using the one-fourth sections of the entire section of a beet, as in the two preceding experiments, 10 comparable pieces whose weight was approximately 13 gm. each were cut from ½-inch-thick sections of large beets, as shown in Figure 1. This was done in order to obtain pieces of similar anatomical and chemical composition and for a more accurate control of the tissue variability within the beet. The tissue near the lateral grooves was especially avoided because of its different composition and character.

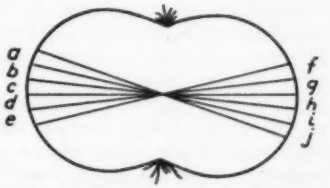

FIGURE 1.—Cross section of large sugar beet, showing method used in experiment 4 of cutting samples (*a* to *f*) from comparable regions

The two procedures explained under experiment 2 were employed.
The inoculated sections were placed on a raised glass platform in a covered glass moisture chamber. Moistened filter paper supplied the necessary humidity. The moisture chambers were then placed in the different control chambers at the desired temperature.

The number of inoculations made in each of the experiments 1, 2, and 3 is given in Table 3.

In experiment 4, four lots of beet sections, of 48 sections each, were inoculated with the four fungus strains 252, 259, 260, and 261, respectively. Each lot of inoculated sections was then subdivided into four lots of 12 sections each, which were held in the control chambers at 10° C. for 12, 24, 36, and 48 days, respectively.

TABLE 3.—*Number of whole beets or sections of beets inoculated with various strains of Phoma betae and placed in control chambers at various temperatures for experiments 1, 2, and 3*

Strain No.	Inoculated beets or sections [a] held at indicated temperature (° C.) in experiments 1, 2, and 3												Total beets used
	1°			5°			10°			15°			
	1	2	3	1	2	3	1	2	3	1	2	3	
252	4	20	24	4	20	24	4	20	24	10	20	24	198
259	4	20	24	4	20	24	4	20	24	10	20	24	198
260	4	20	24	4	20	24	4	20	24	10	20	24	198
261	10	20	24	10	20	24	12	20	24	6	20	24	214

[a] Individual beets were inoculated in experiment 1; in later experiments, sections of beets were used.

The controlled temperatures used in these experiments were made possible by placing temperature-control chambers in cold-storage rooms of a commercial ice and storage company at Salt Lake City, Utah. Each of the temperature-control chambers had a capacity of 5,832 cubic inches and was well insulated. Each chamber had a separate temperature-control device, which consisted of an accurate thermoregulator and a magnetic switch for making and breaking the heating-element circuit. The temperatures were controlled to within $\pm 0.3°$ C. Air temperatures in these control chambers were adjusted to 1°, 5°, 10°, and 15°, the range approximating rather closely actual field-storage conditions.

Student's methods[6] were used for most of the statistical calculations and the probabilities given as values of P.

The probable errors of the mean for the dimensions of the spores were calculated by Bessel's formula, and the probable errors of the differences were calculated by the usual formula, $\pm \sqrt{(E_1)^2 + (E_2)^2}$.

RESULTS OF EXPERIMENTS

Since the pathogenicity of the four strains of *Phoma betae* had been carefully tested shortly after the original cultures were isolated, it was deemed sufficient to make reisolations from diseased beets or sections of beets only in experiments 2 and 3 to serve as checks. The reisolations were made on prune-agar plates by incubating thereon small pieces of beet tissue, part of which gave distinct evidence of infection, although all were cut under aseptic conditions. The strains of the organism were again recovered in pure culture, as indicated in Table 4.

As the results of the first experiment were not used in the primary calculation, it was deemed advisable to present them separately. Table 5 gives the weight in grams of diseased tissue for all the beets inoculated in experiment 1. The results are grouped according to the strain used for inoculation and the temperature at which the inoculated beets were stored. These results indicate differences in the rate of decay caused by the different strains and also strong differential response to the various temperatures.

[6] FISHER, R. A. STATISTICAL METHODS FOR RESEARCH WORKERS. 239 p., illus. Edinburgh and London. 1925.

TABLE 4.—*Reisolations from parts of beets which had been inoculated with various strains of Phoma betae and held at various temperatures in experiments 2 and 3*

Experiment No. and storage temperature (° C.)	Reisolations			
	Total	Negative	Positive	
Experiment 2:	*Number*	*Number*	*Number*	*Per cent*
1°	51	1	50	98
5°	52	1	51	98
10°	55	8	47	85
15°	62	19	43	69
Experiment 3:				
1°	64	3	61	95
5°	66	5	61	92
10°	73	16	57	78
15°	68	8	60	88

TABLE 5.—*Effects of inoculating sugar beets with various strains of Phoma betae and storing for 41 days under different temperatures in experiment 1*

STRAIN 252

Beet No.	Weight of total, healthy, and diseased tissue of inoculated beets stored at temperature (° C.) indicated											
	1°			5°			10°			15°		
	Total	Healthy	Diseased	Total	Healthy	Diseased	Total	Healthy	Diseased	Total	Healthy	Diseased
	Gms.	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*
1	285	260	25	360	310	50	370	300	70	413	368	45
2	445	420	25	465	430	35	190	140	50	68	0	68
3	315	275	40	310	280	30	285	270	15	345	325	20
4	470	450	20	411	390	21	225	185	40	122	72	50
5										132	90	42
6										355	325	30
7										300	185	115
8										211	150	61
9										235	210	25
10										160	150	10
Total	1,515	1,405	110	1,546	1,410	136	1,070	895	175	2,341	1,875	466
Average	378.75	351.25	27.50	386.50	352.50	34	267.50	223.75	43.75	234.10	187.50	46.60
Diseased			*P. ct.* 7.28			*P. ct.* 8.80			*P. ct.* 16.36			*P. ct.* 19.91

STRAIN 259

			Gms.			*Gms.*			*Gms.*			*Gms.*
1	405	370	35	185	150	35	150	90	60	120	0	120
2	173	165	8	275	250	25	180	90	90	170	50	120
3	275	230	45	170	140	30	120	25	95	54	40	14
4	380	330	50	190	160	30	287	200	87	88	40	48
5										155	110	45
6										350	190	160
7										120	50	70
8										60	25	35
9										50	10	40
10										135	60	75
Total	1,233	1,095	138	820	700	120	737	405	332	1,302	575	727
Average	308.25	273.75	34.50	205	175	30	184.25	101.25	83	130.20	57.50	72.70
Diseased			*P. ct.* 11.19			*P. ct.* 14.63			*P. ct.* 45.05			*P. ct.* 55.84

TABLE 5.—*Effects of inoculating sugar beets with various strains of Phoma betae and storing for 41 days under different temperatures in experiment 1*—Contd.

STRAIN 260

Beet No.	Weight of total, healthy, and diseased tissue of inoculated beets stored at temperature (° C.) indicated											
	1°			5°			10°			15°		
	Total	Healthy	Diseased	Total	Healthy	Diseased	Total	Healthy	Diseased	Total	Healthy	Diseased
	Gms.	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*
1	260	240	20	370	350	20	232	220	12	402	330	72
2	355	330	25	385	360	25	515	470	45	360	260	100
3	228	225	3	417	410	7	145	125	20	60	35	25
4	278	270	8	240	230	10	162	140	22	155	145	10
5										115	70	45
6										210	140	70
7										170	120	50
8										110	15	95
9										70	60	10
10										68	60	8
Total	1, 121	1, 065	56	1, 412	1, 350	62	1, 054	955	99	1, 720	1, 235	485
Average	280. 25	266. 25	14	353	337. 50	15. 50	263. 50	238. 75	24. 80	172	123. 50	48. 50
Diseased			*P. ct.* 5. 00			*P. ct.* 4. 39			*P. ct.* 9. 39			*P. ct.* 28. 20

STRAIN 261

			Gms.			*Gms.*			*Gms.*			*Gms.*
1	170	160	10	600	580	20	470	440	30	235	200	35
2	175	165	10	710	680	30	285	250	35	345	300	45
3	385	370	15	165	160	5	330	300	30	245	185	60
4	115	110	5	125	120	5	277	220	57	270	160	110
5	132	110	22	200	180	20	515	480	35	210	170	40
6	465	430	35	215	190	25	338	310	28	270	230	40
7	248	240	8	270	230	40	530	500	30			
8	179	170	9	214	210	4	580	530	50			
9	345	330	15	178	170	8	335	310	25			
10	285	260	25	315	290	25	415	330	85			
11							480	460	20			
12							245	210	35			
Total	2, 499	2, 345	154	2, 992	2, 810	182	4, 800	4, 340	460	1, 575	1, 245	330
Average	249. 90	234. 50	15. 40	299. 20	281	18. 20	400	361. 67	38. 33	262. 50	207. 50	55
Diseased			*P. ct.* 6. 16			*P. ct.* 6. 08			*P. ct.* 9. 58			*P. ct.* 20. 95

In experiment 1 whole beets were used for each inoculation. Because of the great irregularity in the weights of diseased tissue from different beets when subjected to the same organisms and temperature, only sections of beet tissue (fig. 1) were used for the later experiments, thus eliminating differences due to variability among individual beets. While this procedure reduced the irregularities in the results, statistical methods showed other variabilities which it is believed might have been due to faulty technic in procuring a uniform quantity of inoculum throughout the tests or to variability in conditions of storage.

In experiments 2 and 3 strains 252 and 259 were found to have the highest relative rate of decay and strain 260 the lowest rate of decay, as shown in the following comparison of average weights of diseased tissue per day for each strain of *Phoma betae*.

Strain No.	Diseased tissue (gm.)
252	0.4958
259	.4968
260	.2242
261	.4070

The following probability values were calculated[7] for differences in decay produced by each pair of strains of *Phoma betae* at all temperatures (1°, 5°, 10°, and 15° C.) used in experiments 2 and 3.

Pairs of strains	Probability value (*P*)
252 and 259	0.45
252 and 260	.01
252 and 261	.18
259 and 260	.01
259 and 261	.15
260 and 261	.01

These data indicate that strain 260 was the least aggressive and that each one of the three others was significantly different from it. The differences in respect to aggressiveness between strains 252, 259, and 261 are not clearly significant. When the data for these organisms are analyzed for the individual temperatures, as shown in Table 6, the differences are more clearly apparent. Strain 252 was not significantly different from 259 or 261 in amount of decay produced, but was significantly different from 260. Strain 259 was not significantly different from 261, but was markedly different from 260. The amount of decay produced by strains 260 and 261 was significantly different at all temperatures investigated.

TABLE 6.—*Probability values for the differences in decay produced by each pair of strains of Phoma betae used in experiments 2 and 3*

Pairs of strains	Temperature (° C.)	Probability value (*P*)[a]		Pairs of strains	Temperature (° C.)	Probability value (*P*)[a]	
		Experiment 2	Experiment 3			Experiment 2	Experiment 3
252 and 259	1	0.59	0.84	259 and 260	1	0.06	0.01
Do	5	.70	.29	Do	5	.04	.01
Do	10	.55	.38	Do	10	.01	.01
Do	15	.61	.11	Do	15	.06	.01
252 and 260	1	.01	.01	259 and 261	1	.54	.04
Do	5	.05	.01	Do	5	.44	.19
Do	10	.02	.01	Do	10	.02	.27
Do	15	.10	.16	Do	15	.04	.18
252 and 261	1	.36	.04	260 and 261	1	.01	.01
Do	5	.69	.02	Do	5	.01	.01
Do	10	.03	.13	Do	10	.02	.01
Do	15	.18	.82	Do	15	.03	.03

[a] A probability value of 0.05 for *P* was considered the limit of significance.

Data showing the effect of temperature on the amount of tissue destroyed by each strain are given in Table 7. These results indicate that the weight of tissue destroyed at 5° C. was in no case significantly different from that destroyed at 1°. Strain 261 did not destroy significantly more tissue at 10° than at 1° or 5°. In all other cases, except strain 260 at 5° to 10°, the weights of tissue destroyed by each strain were significantly different for each 5 degrees change of temperature.

[7] A probability value of 0.05 for *P* was considered the limit of significance.

TABLE 7.—*Probability values for differences in weight of tissue destroyed by each strain of Phoma betae at temperatures of 1°, 5°, 10°, and 15° C. in experiments 2 and 3*

Temperature (° C.)	Strain No.	Probability value (P)[a]	Temperature (° C.)	Strain No.	Probability value (P)[a]
1° and 5°	252	0.12	5° and 10°	252	0.04
Do	259	.53	Do	259	.02
Do	260	.18	Do	260	.07
Do	261	.51	Do	261	.15
1° and 10°	252	.01	5° and 15°	252	.02
Do	259	.01	Do	259	.01
Do	260	.04	Do	260	.01
Do	261	.16	Do	261	.01
1° and 15°	252	.01	10° and 15°	252	.04
Do	259	.01	Do	259	.02
Do	260	.01	Do	260	.01
Do	261	.01	Do	261	.01

[a] A probability value of 0.05 for *P* was considered the limit of significance.

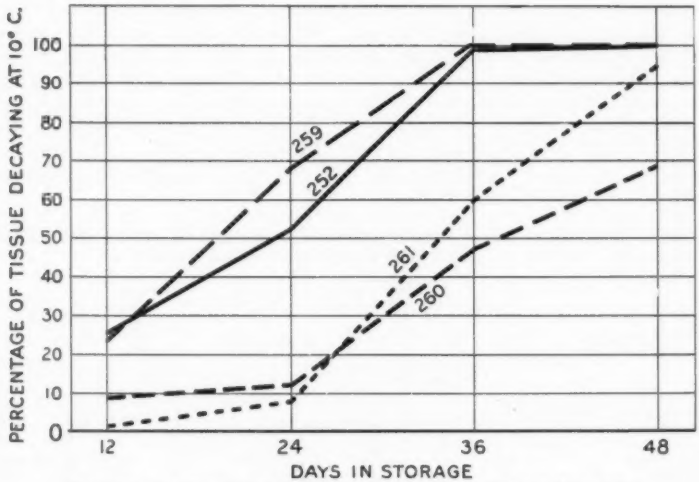

FIGURE 2.—Relative progress of decay in each of the four strains of *Phoma betae*

The rate of decay, based on the average of results obtained in experiment 4, is expressed as curves in Figure 2 for strains 252, 259, 260, and 261, respectively. The curve of strain 252 is similar to that of strain 259, whereas the curve of strain 260 resembles that of strain 261. These curves confirm the statistical results presented in Tables 5, 6, and 7, and also show that strains 252 and 259 destroy sugar-beet tissue more rapidly than do strains 260 and 261. Strains 252 and 259 destroyed practically all the available tissue within 36 days, but strains 260 and 261 did not. Strain 260 was again shown to be the least aggressive. Strain 261 attacks slowly, as does 260, but progresses at a rapid rate, approaching the curves of strains 252 and 259 at about the forty-eighth day. These curves, together with the fact that the experiments deal with the relative rate of decay produced by the strains during long storage periods, show why it was not possible to establish significant differences between strains 261 and 252 or between

strains 261 and 259. The curves indicate a probable difference in aggressiveness for strain 261 and show the advantage of running such tests at varied temperatures and for various lengths of storage periods.

DISCUSSION

Phoma betae is undoubtedly the most important wound parasite found in stored sugar beets in the Western States, particularly in northern Utah. While no figures are available as to the economic losses resulting from the action of this fungus, it is safe to assume that the damage incurred from year to year has generally reached significant proportions. Reduction in the total extractable sucrose, occasioned by rotting of the healthy tissues, and in the weight of the stored beets constitutes the main source of loss.

The experimental results herein reported show that temperature is one of the most important ecological factors associated with decay of beets in storage. In general, low temperatures retard the penetration of the fungus into exposed crown tissues and the higher temperatures favor it. It would therefore seem advisable to delay until the advent of cooler weather the piling of beets in those localities where high temperatures prevail. The length of the storage period may determine in large measure whether or not losses will become significant, according to the rate of decay produced by the strains of *Phoma betae* present in the storage pile. Although only four strains of the fungus were used in the experiments reported in this paper, the senior writer has isolated from various sources more than 20 strains that gave evidence of morphologic variation. Since marked differences in size of spores were found in two of the strains used in these experiments and since similar variations in size of spores have been noted in other strains, there is reason to believe that a number of distinct strains exist. The results of the present investigation indicate wide differences in the capacity of these strains to decay sugar-beet tissue.

SUMMARY

The relation of temperature and length of storage to rotting of sugar beets by the fungus *Phoma betae* (Oud.) Frank has been investigated. Distinct morphologic differences have been found between two of the strains used.

Four strains of the fungus were studied under controlled conditions to determine their relative capacity for producing decay. The data indicate that the four strains of *Phoma betae* tested vary in the rate at which they destroy beet tissue, the differences amounting to as much as 50 per cent but varying according to the temperature and period of storage.

Increase in temperature during storage favored increased metabolism of the fungus, with attendant increase in rotting of tissues. In general, significant differences were noted for each change of 5° C. in temperature.

Strains 252 and 259 were found to possess similar aggressiveness, although they differed significantly in morphology. Both differed markedly from strain 260 in the amount of decay produced. Strains 260 and 261, the morphology of which was not studied, were found to be significantly different in their ability to produce decay. It is therefore believed that distinct strains of *Phoma betae* exist.

ROOT CONSTRICTION OF COTTON PLANTS IN THE SAN JOAQUIN VALLEY OF CALIFORNIA[1]

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INTRODUCTION

Cotton plants may be severely stunted, or even killed, when their roots are choked in hard soil. The injury occurs a little below the surface of the ground, where the soil becomes dry and hardens around the small seedling taproot, thus preventing further expansion. As the plant grows the stalk bulges out over the hard soil and forms an enlarged calloused base, contrasting with the threadlike taproot firmly encased in the hard soil. The plant reaches a stage of development where such constriction of the root causes it to wilt rather suddenly and it will die if the condition is not relieved in a short time.

In Texas, instances of root constriction have been reported in which cotton plants growing in heavy clay soils were strangled as a result of root constriction when the soil was compacted by continuous rain or excessive irrigation.[2] A similar effect was observed by the writer at the United States cotton field station, Shafter, Calif., in 1930. In this case the constriction was caused by withholding water early in the season, thus allowing the soil to become dry and hard near the surface.

The disorder as noted in Texas was recorded as a new disease and named "root strangulation"; but since it is neither physiological nor parasitic, it is hardly to be reckoned as a disease unless the word is used in its broadest sense, to include any departure from normal structure or function. Cook,[3] in discussing leaf cut, or tomosis, a common disorder of cotton seedlings, recognizes a class of ecological disorders intermediate between physiological diseases and mechanical injuries or traumatisms. To this intermediate class the strangulation of plants by root constriction may be added, though the causal factors are purely mechanical.

OBSERVATIONS AND STUDIES

At Shafter, Calif., several dead plants were found in plots of cotton that showed no general indications of stress conditions. When examined the roots of these plants were found to be severely constricted just under the surface mulch where they entered the firmer soil. In most cases the root just above the constriction was enlarged beyond the normal size at the base of the stalk, and calloused. The reduction from these large calloused bases to the constricted root below was usually very abrupt. Often a reduction from a stem 1.5 to 2 cm. in diameter to a root about 1 mm. in diameter would occur in the space of 1 cm. or less.

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[1] Received for publication July 29, 1931; issued February, 1932.

[2] ANONYMOUS. [COTTON PLANTS ARE STRANGLED TO DEATH BY HARD, DRY CLAY IN A NEW DISEASE OF COTTON . . . (Item) Science (n. s.) 71 (1847): XIV. 1930.

[3] COOK, O. F. LEAF-CUT, OR TOMOSIS, A DISORDER OF COTTON SEEDLINGS. U. S. Dept. Agr., Bur. Plant Indus. Circ. 120: 29-34. 1913.

The constrictions of the roots were usually from 3 to 6 inches or more in length, extending through the extremely hard dry soil near the surface. Beneath this hard dry soil layer the roots appeared normal in every respect. In many cases there were no lateral roots near the surface and the plants were merely resting on flat, calloused bases. In such cases the plants were kept erect largely by the tension of the threadlike taproots that held these large calloused bases firmly against the hard soil. When the plots were irrigated many such plants fell prostrate as the soil softened and gave way under the bases.

The first observations of the disorder at Shafter were recorded July 2, 1930, the condition appearing in a series of plots that were planted April 21. These plots had been irrigated on April 15 by flooding and were harrowed lightly before planting. On June 13 the stand was thinned to about 12 inches between plants, and this operation was followed by a shallow cultivation, leaving a surface mulch from 2 to 3 inches deep. The average plants in the plots were about 12 to 15 inches high and had been flowering for several days when the first dead ones were observed. The dead and severely wilted plants were slightly smaller than the average. Further investigations showed that most of the plants were more or less constricted, and many of the smaller ones were observed to show slight symptoms of a deficient water supply. In the following days more of these plants wilted and died rather suddenly, and the condition became so severe that the plots were irrigated on July 8. Several plants that were severely wilted immediately before irrigation were tagged for further study. Some of these recovered very slowly from their wilted condition, while others regained turgidity soon after irrigation but remained a dull bluish color for several days, indicating water stress. Only a few plants failed to recover, and these were practically dead when irrigated.

Several of the tagged plants were removed July 10, two days after irrigation, by digging them carefully and washing the soil from the roots. All of these plants had badly constricted taproots and very few old lateral roots near the surface, but they had many white rootlets springing from the bases of the stalks and taproots. These rootlets ranged in length from very short stubs to more than one-fourth inch, as shown in Figure 1.

Additional specimens of the plants that were severely wilted before irrigation were removed July 22. Most of these recovered completely, and in every case in which the top of the plant had recovered the taproot was filled out to normal size. A few of the tagged plants were left undisturbed throughout the season. These showed no ill effect of the early constriction but developed normally and produced good crops of cotton.

Figure 2 shows a plant severely wilted from root constriction, in comparison with an adjacent normal plant. When the plot was irrigated a few minutes after this photograph was taken the wilted plant fell because the soil softened beneath its base. Other plants in the same plot that were wilted to about the same extent immediately before irrigation made recoveries, some apparently complete, others only partial. The degree of wilt represented by the wilted plant in Figure 2 appeared to be about the limit of stress from which a plant could recover to normal.

FIGURE 1.—Parts of constricted cotton plants two days after irrigation, showing the development of white rootlets and the quick response of the plants to irrigation. (Natural size)

PHOTOGRAPHIC RECORDS

In a further study of this disorder, in another set of plots, two series of natural-size photographs were made of root-constricted plants, showing portions of the taproots before and after irrigation. The first series was begun July 23 in a plot of cotton that was planted May 24, and the second series was begun August 15 in a plot planted June 7. Neither of these plots had been irrigated after planting, previous to the beginning of the studies. A group of three plants in each plot was used for study. The first group will be referred to as series 1 and the second group as series 2. Photographs are shown of only one plant in each group. In series 1 the photographs of plant

FIGURE 2.—Constricted cotton plant (at right) showing severe condition of wilt, in comparison with an adjacent normal plant. This severely wilted condition is reached in a few hours after wilting starts. Such plants do not recover at night

1 typify all three plants studied. In series 2 only one of the three plants survived.

SERIES 1

The upper portions of the taproots of the three plants in series 1 were photographed July 23. This was done by digging a trench about 8 to 10 inches deep close to the plants and washing away the soil from one side of the taproots by means of a small pressure tank and hose. Natural-size photographs were taken as soon as the taproots were clearly exposed. The soil was then carefully replaced about the roots and the plot irrigated.

The roots of these plants were again photographed in a similar manner on August 8 for comparison with the photographs taken on July 23. Figure 3 shows the condition of the taproot of plant 1

of this series at the time of the first and second exposures. These observations merely confirm the result of the more casual determinations already made showing that the plants were able to fill out the

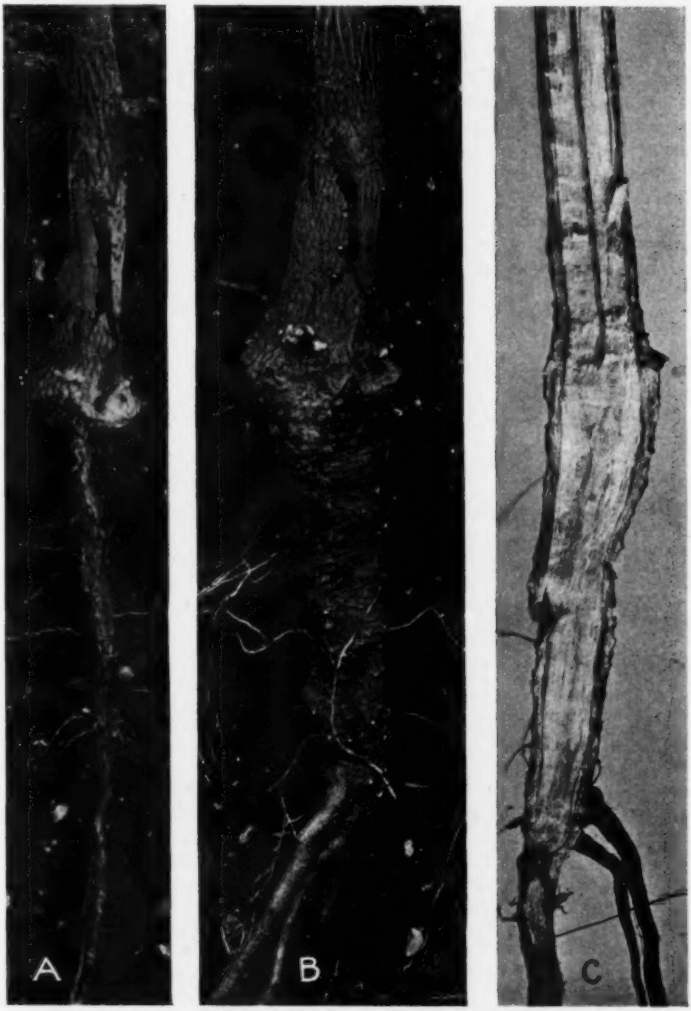

FIGURE 3.—Three views of base of stalk and taproot of plant 1, series 1: A, Severely constricted taproot as it appeared before irrigation, July 23; B, taproot showing recovery from constriction, August 8, 16 days after irrigation; C, longitudinal section of the root after recovery. (Natural size)

constrictions of the roots in a very short time after irrigation. Each of the three plants under observation showed severely constricted roots before irrigation and a complete recovery 16 days after irriga-

tion. The plants were removed from the field on August 8, and the taproots were split in longitudinal sections to show the newly developed wood. (Fig. 3, C.) No lines of demarcation were visible between the old wood of the constricted root and the new wood formed after irrigation.

SERIES 2

In series 2, consisting of three plants, the roots were first exposed for photographing on August 15, primarily to determine the rate of recovery of the constricted roots of plants that recovered promptly aboveground. These plants were in a plot that was planted June 7 and received no irrigation after seeding. Many plants in this plot were dead or dying from root constriction when the first exposure was made. Figure 4 shows a section of a row near the location of the three plants of this series, taken just before irrigation, showing plants in several stages of wilt caused by root constriction. This plot was

FIGURE 4.—Section of row near the location of the three plants selected for study in series 2. This photograph, taken just before the first irrigation after planting, shows several plants severely wilted from root constriction, together with normal-appearing plants

irrigated August 15 immediately after the first set of photographs was taken.

Plant 1 of this series was turgid and green when photographed, but wilted soon afterward and did not make a complete recovery. Plants 2 and 3 were slightly wilted at 9 a. m., before the roots were disturbed. Plant 2 failed to recover and was dropped with plant 1 from this investigation, leaving only plant 3 to be studied for the rate of root expansion. This plant recovered from wilting promptly and assumed a normal appearance aboveground a few days after irrigation. A second exposure was made of this plant on August 21 and a third exposure on August 26. Figure 5 shows the condition of the taproot at the time of the first, second, and third exposures, respectively. Some idea of the rapid development that takes place in constricted roots after irrigation may be had by comparing these views. The rapid development of lateral roots near the surface of the ground appears remarkable.

In Figure 5, A, which shows the plant before irrigation, it may be seen that there were no lateral roots near the surface at the time of the first exposure. In B, which was taken six days after the first exposure and irrigation, many small white lateral roots are shown. These roots were very tender and some were broken; others were exposed entire, but care was taken not to disturb them more than was

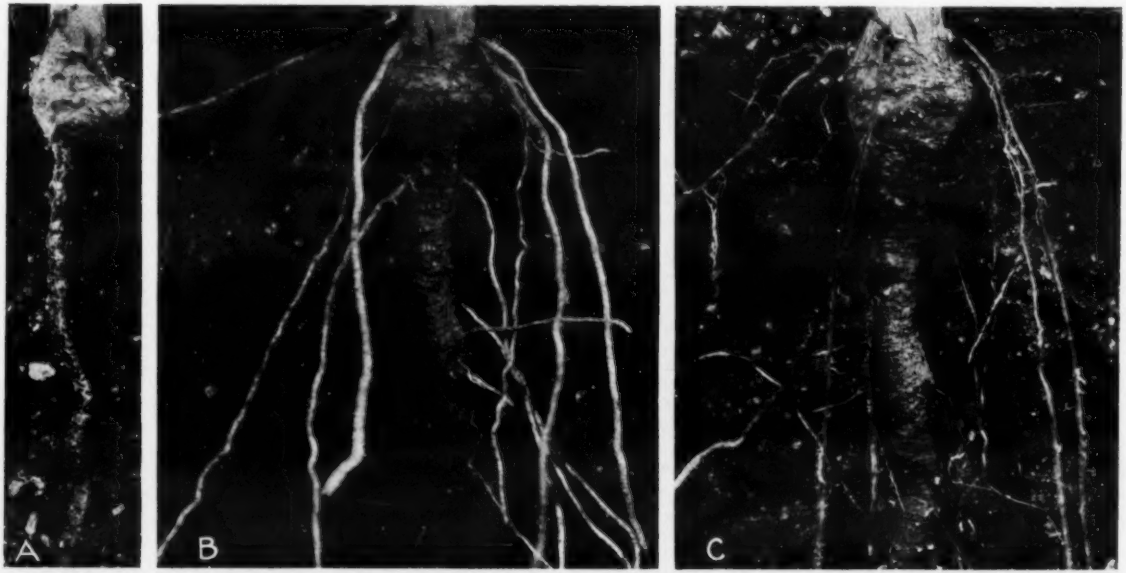

FIGURE 5.—Base of stalk and roots of plant 3, series 2, showing rapid development after irrigation, (about seven-eighths natural size): A, View August 15, before irrigation, showing taproot severely constricted with no lateral roots near the surface; B, view August 21, six days after irrigation, showing taproot greatly increased in diameter, and development of many small white lateral roots, some of them more than 6 inches long; C, view August 26, five days after B and 11 days after A, showing taproot practically normal in size and lateral roots of tough woody structure with numerous secondary branches

necessary, since further study of the plant was desirable. Figure 5, C, which was taken five days after B and 11 days after A, shows these laterals to be of tough, woody structure, with many small secondary branches.

Several neighboring plants were removed from the plot August 21 and cross sections were made of the taproots through the constriction. A small hard center of old wood was clearly distinguishable in these sections, with a large ring of clear, semitransparent wood and rather heavy bark. The proportions of old wood, new wood, and bark on an average root were, respectively, three thirty-seconds, three thirty-seconds, and one-sixteenth of an inch. The new wood was very soft, and the greater part of it could be scraped off easily with the thumb nail into a clear jellylike mass. Similar cross sections of roots removed August 26 showed no definite lines of demarcation between the old and new wood, but the cambium was very active and a thin outer portion of the wood was rather soft.

DISCUSSION

Constriction of taproots was found to be general at the United States cotton field station in plots that were not irrigated for a long time after planting, and in many cases it became necessary to irrigate before the plants had reached a stage of development where irrigation appeared desirable, except as a measure to prevent some losses in seedling stand. Some plots at the station were irrigated early in the season during the seedling stage of the plants, and the roots of these plants developed normally, while the roots of plants in adjoining plots that were not irrigated became constricted. The general practice among cotton growers in the San Joaquin Valley is to irrigate freely rather early in the season, in order to develop a large plant, and then to stop irrigation in an effort to force maturity of the crop. While this practice is not considered a good cultural method, it prevents root constriction and is probably the reason why the disorder has not been reported heretofore. The objection to irrigating early in the season before the cotton has reached the flowering stage is that the plants may grow too rank and fail to mature as large a crop of bolls as they would otherwise. Moreover, the plants that have too much water at first may develop shallow root systems and are therefore likely to suffer in dry weather, so that both the quality of the fiber and the yield may be impaired. It appears that root constriction may interfere somewhat with the application of improved cultural methods on the sandy soils that become very hard when dry; but since the injury is not permanent and does not appear to affect the later development of the plants in any way, it is probably of minor importance.

SUMMARY

Cotton plants grown at the United States cotton field station at Shafter, Calif., in 1930 were observed to wilt and die suddenly as a result of taproot constriction. The soil in which these plants were grown is a light sandy loam that becomes very hard when dry, and the constricted plants were found in each case in plots that had not been irrigated after planting.

Irrigation corrected the condition that caused constriction, and the plants that were not too severely injured recovered after irrigation and developed into normal, well-fruited plants.

Photographs of constricted roots taken before and after irrigation show that the plants made a rapid recovery from the disorder as soon as the cause was removed.

In one series of photographs the development of new lateral roots near the surface of the ground is shown. A plant having no surface lateral roots before irrigation is shown on the sixth day after irrigation with numerous small, white lateral roots, some of them more than 6 inches long. Eleven days after irrigation these new laterals had greatly increased in length, were tough and woody, and had many branches.

In cross sections of constricted roots made during the rapid growth after irrigation a large ring of soft semitransparent wood tissue was observed between the old wood and the bark. Six days after irrigation this new wood tissue could be scraped off with little effort into a clear, jellylike mass, but 11 days after irrigation no lines of demarcation were perceptible to the naked eye between the new and the old wood.

The disorder is probably of little importance to the cotton grower under present conditions, but it may interfere with the utilization of the best cultural methods unless some practical method other than irrigation is devised to correct the conditions causing it.

HETEROTHALLISM AND HYBRIDIZATION IN TILLETIA TRITICI AND T. LEVIS [1]

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INTRODUCTION

The most destructive disease of wheat in the Pacific Northwest is bunt or stinking smut. Seed treatment is not entirely effective on fall-sown wheat because of soil infestation. Consequently, the most promising means of control appears to be the development and use of resistant varieties.

A number of varieties apparently resistant to bunt have been developed. For a number of years these varieties maintained their resistance, but they are now being attacked with increased severity from year to year. Gaines [2] has shown that these varieties are being attacked by new forms of *Tilletia tritici* (Bjerk.) Wint. and *T. levis* Kühn. Prior to 1918 only *T. tritici* was known to occur in the State of Washington (*10*).[3] By the inoculation of selected varieties with a number of field collections, Gaines in 1928 found that the resistant varieties had maintained their resistance to the old form of *T. tritici*, but that two additional forms of *T. tritici* and four forms of *T. levis* were present in this region.

This increasing amount of smut in previously resistant varieties showed the importance of a thorough consideration of the rôle of physiologic specialization in a breeding program and emphasized the necessity for obtaining fundamental information concerning the origin of these new forms as well as their prevalence and distribution.

The occurrence of physiologic specialization in the cereal smuts has been repeatedly demonstrated. According to Stakman (*16*), physiologic forms may be differentiated by (1) cultural characters, (2) physicochemical reactions, (3) morphology, and (4) pathogenicity. Pathogenicity as shown by varietal reaction has been the criterion most widely used for differentiation and is of most importance from the standpoint of the plant breeder.

Usually little if any effort has been exercised by the various investigators to insure the purity of the physiologic forms of smut with which they have worked. The usual procedure has been to obtain collections from various localities or from resistant varieties and test these on a number of varietal host testers directly, or after the collection has been increased on a susceptible host. In some instances an attempt has been made to purify the collections by increasing them on their respective differential hosts, i. e., the variety that each collection is able to infect but that is not attacked by other collections.

[1] Received for publication July 16, 1931; issued February, 1932. The investigations herein recorded were conducted by the Division of Cereal Crops and Diseases in cooperation with the Washington and Oregon Agricultural Experiment Stations.

[2] GAINES, E. F. WHY SMUT HAS BEEN INCREASING. U. S. Dept. Agr., Off. Coop. Ext. Work, Ext. Path. 6 (2): 14-15. February, 1928. [Mimeographed.]

[3] Reference is made by number (italic) to Literature Cited, p. 58.

Obviously, this method would strain out those forms that were unable to infect this variety. However, it would give no assurance that the collection had been reduced to a single physiologic form, as it is possible that a number of forms or hybrid forms exist that are able to attack any given variety. In order to study the importance of mutation and hybridization in the development of pathogenically different physiologic forms, it is imperative that the work be started with lines as pure as possible.

The present investigation was undertaken to study the pathogenicity of single and paired monosporidial cultures of *Tilletia tritici* and *T. levis* and thus learn as much as possible concerning the nature of infection, the importance of hybridization in the development of new forms, and the possibility of developing improved methods for the purification of the physiologic forms.

HISTORICAL REVIEW

The fusion of sporidia in certain smuts has been observed by a number of investigators. Kniep (*11*) in 1919, working with *Ustilago violacea* Pers., was the first to show that this fusion occurs only between certain sporidia and that therefore the smut is heterothallic. Since then a number of species of the Ustilagineae have been studied and found to be heterothallic, except for Christensen's (*2*) report of infection by monosporidial lines of *U. zeae* (Beckm.) Ung. Zillig (*17*) confirmed the work of Kniep and further showed that conjugation occurred between sexual strains of different physiologic forms. Kniep (*12*) observed conjugation between the sporidia of a number of smooth-spored species of Ustilago and also between different echinulate-spored species and reticulate-spored species. He also observed conjugation between sporidia of certain smooth-spored and echinulate-spored species, but observed none between sporidia of reticulate-spored and those of either smooth-spored or echinulate-spored species. Dickinson (*5*) observed hyphal fusion in the host tissue between monosporidial cultures of *U. hordei* (Pers.) Kell. and Sw. and *U. nuda* (Jens.) Kell. and Sw., but did not permit his plants to grow to maturity. Apparently interspecific fusion is common in the smuts. However, none of these investigators completed their tests. They did not report whether the interspecific crosses were able to cause infection, as shown by the production of mature chlamydospores in the host plant. This information is exceedingly important from the point of view of the investigator interested in the origin and development of physiologic forms. Hanna and Popp (*9*) found that *U. avenae* (Pers.) Jens. and *U. levis* (Kell. and Sw.) Magn. were heterothallic and that a monosporidial culture of one species mated readily with one of opposite sex of the other species. From this mating a smutted panicle was produced which was somewhat intermediate in appearance between the loose and covered types, and the spores were echinulate.

A number of investigators have attempted to infect wheat plants with cultures of the bunt organism. Sartoris (*15*) failed to obtain infection, although he grew his wheat in large flasks containing a culture of the organism. Kienholz and Heald (*10*) likewise failed to obtain infection when the seed was inoculated with cultures derived

from the chlamydospore mass. Bodine and Durrell (*1*) obtained infection by inoculating wheat seedlings with cultures derived from secondary sporidia produced by a chlamydospore mass culture.

FORMATION OF SPORIDIA

The chlamydospores of *Tilletia tritici* and *T. levis* germinate by forming a promycelium into which the protoplasm of the spore passes. As the promycelium elongates, the protoplasm is confined to the upper portion, and septa are laid down as the basal portion is evacuated. At the tip of the promycelium is produced a crown of 8 to 24 long slender sporidia, which in this paper are termed "primary" sporidia. All cultures used in the tests reported in this paper were derived from single primary sporidia. These primary sporidia commonly fuse to form the H-shaped sporidia. Usually both the single primary sporidia and the H-shaped sporidia germinate by sending out a single germ tube or mycelial filament which may or may not produce sickle-shaped secondary sporidia. However, both types of primary sporidia occasionally produce more than one germ tube.

MATERIALS AND METHODS

The monosporidial cultures used in these tests were derived from three strains of *Tilletia tritici* and two of *T. levis* that had been collected by E. F. Gaines in the Pacific Northwest and differentiated by their action on wheat varieties.

The methods of making single sporidial isolations as described by Dickinson (*4*) and Hanna (*7*) for smuts of the Ustilago type were tried but were not satisfactory. Single spores of *Tilletia tritici* and *T. levis* did not germinate normally on culture media, although in mass normal germination was obtained. Kienholz and Heald (*10*) found this to be true when they attempted to start cultures with single chlamydospores. Furthermore, the sporidia were produced in a crown at the tip of the promycelium and were difficult to separate. Until fully mature they could not be separated from the tip of the promycelium without injury, and when mature many of them were fused to form the H-shaped sporidia.

Two fairly satisfactory methods were used in isolating single sporidia. In both methods the smut ball was carefully removed from the head, dipped momentarily in 95 per cent alcohol, flamed, and then crushed with forceps over a Petri dish of nonnutrient agar. This was incubated at 15° C. from four to seven days until mature primary sporidia were formed but before there was much germination of the primary sporidia.

In the first method of isolation a suspension was made of the sporidia in sterile water and this suspension streaked with a platinum loop on a thin layer of nonnutrient agar in a Petri dish. The sporidia germinated in from 6 to 24 hours by sending out germ tubes into which the entire protoplasmic contents of the sporidium passed. The sporidia that were sufficiently isolated were then transferred to Thaxter's potato hard agar. This was accomplished with a glass needle drawn out to a diameter equal to two-thirds the length of a primary sporidium and rounded at the tip so as not to cause injury. The needle was sterilized by dipping it in alcohol. The sporidium

was removed, under the 16-mm. objective of a microscope, by touching the needle to the evacuated portion of the sporidium and germ tube, and was deposited on the nutrient agar by drawing the needle across the surface at an oblique angle. The sporidium was invariably deposited at the beginning of the stroke and, if the needle was not forced too deeply into the agar, could be readily seen and the isolation verified by looking through the bottom of the Petri dish with the 16-mm. objective.

The second method was somewhat similar to the first in that all the operations were performed under the 16-mm. objective. All the sporidia were removed with the glass needle from the tip of promycelium as soon as mature and transferred to a Petri dish of nonnutrient agar. They were then separated from one another with a glass needle and transferred to nutrient agar one at a time.

The subsequent treatment was the same in both methods. The isolated sporidia were incubated from 7 to 10 days at 15° C. At the end of the incubation period the sporidia that were growing had usually developed a colony about 0.1 cm. in diameter and were visible to the naked eye. The colony was then transferred to a test tube of potato agar containing 1 per cent dextrose. Only 1 per cent was used because a deficiency of sugar in the medium was found to be conducive to the maintenance of that stage of the fungus characterized by the abundant production of secondary sporidia.

Because of the apparent difficulty in obtaining infection from bunt cultures, the following technic was adopted: Prelude wheat, a susceptible spring variety, was soaked for one hour in a 1–400 Uspulun [4] solution, washed thoroughly, and then dried. The seed was germinated in sterile Petri dishes on filter paper. When the germinating sprouts were one-fourth of an inch long they were inoculated by holding the seed between sterile forceps, puncturing the stem near the base with a sterile needle, and working the inoculum into the wound. The inoculated seedlings were again placed on moist filter paper in sterile Petri dishes and incubated four days at 15° C. They were then transferred to cans of sterile soil, kept in temperature tanks maintained at 15° for approximately two weeks, and then transplanted to a bench of steamed soil in a greenhouse maintained at 15° to 17°.

The single primary sporidial cultures tested were paired in all possible combinations, and 15 seedlings were employed in each test. Lights were used in the greenhouse, and the wheat headed about 45 days after it was transplanted to the bench.

EXPERIMENTAL RESULTS

HETEROTHALLISM IN TILLETIA TRITICI

The pathogenicity of 12 single primary sporidial cultures derived from the same wheat head of *Tilletia tritici*, form 3, was tested alone and in all possible paired combinations. Although 15 plants were inoculated in each case, that number seldom survived, owing to the severity of the injury at the time of inoculation. As the object of the test was primarily to determine which matings produced infection, as evidenced by smut balls in the head, the number of surviving plants in cases where infection occurred was not important. However, it is

[4] A commercial seed disinfectant.

recognized that the results in cases in which infection did not occur, even when all inoculated plants survived, while strongly indicative, may not have been conclusive.

It is possible that pathogenicity is not synonymous with sexual compatibility. It may be influenced by physicochemical or other factors entirely separated from sex. However, until more information concerning the relationship of sexuality and pathogenicity becomes available, it seems advisable to consider the ability of two monosporidial lines to produce smut balls in the wheat head as a measure of their sexual compatibility.

The results obtained in pairing 12 single primary sporidial cultures of form 3 of *Tilletia tritici* are presented in Table 1. These show that the species is heterothallic, for in no case was infection produced by a single culture. A number of sex groups appear to be involved. Hanna (*8*) and Christensen (*2*) have shown that this is true in *Ustilago zeae*. Cultures 209 and 213 appeared to belong to one group which when paired with 240, 243, 263, 303, 304, or 306 were able to produce infection. Although 240 and 243 did not cause infection when paired with 213, it is probable, in view of the similarity of reaction of 209 and 213 to the other numbers in this group and because of the small number of plants involved, that they belong to the same group as 263, 303, 304, and 306. Culture 235 produced infection only when mated with 302 and 305, showing the existence of another sexually compatible group. Culture 262 failed to cause infection when paired with any of the other cultures. This indicates either that it had lost its vigor or that another sex group exists.

TABLE 1.—*Results of inoculating Prelude wheat with single and paired cultures derived from single primary sporidia of form 3 of Tilletia tritici*

Culture No.	209	213	235	240	243	262	263	302	303	304	305	306
209	−	−	−	+	+	−	+	−	+	+	−	+
213	−	−	−	−	−	−	+	−	+	+	−	+
235	−	−	−	−	−	−	−	+	−	−	+	−
240	+	−	−	−	−	−	−	−	−	−	−	−
243	+	−	−	−	−	−	−	−	−	−	−	−
262	−	−	−	−	−	−	−	−	−	−	−	−
263	+	+	−	−	−	−	−	−	−	−	−	−
302	−	−	+	−	−	−	−	−	−	−	−	−
303	+	+	−	−	−	−	−	−	−	−	−	−
304	+	+	−	−	−	−	−	−	−	−	−	−
305	−	−	+	−	−	−	−	−	−	−	−	−
306	+	+	−	−	−	−	−	−	−	−	−	−

HYBRIDIZATION OF PHYSIOLOGIC FORMS OF TILLETIA TRITICI

In Tables 2 and 3 are given the results of pairing monosporidial cultures of forms 1 and 2 of *Tilletia tritici* with two cultures of form 3 which were known to be sexually compatible from preliminary work (*6*). Although these tests were limited and none of the four sporidia selected from either form 1 or 2 were compatible with one another, they showed that these forms readily hybridized with form 3. Culture 297 of form 1 caused infection when paired with culture 263 of form 3, and cultures 288 and 293 of form 2 reacted similarly with culture 209 of form 3.

TABLE 2.—*Results of inoculating Prelude wheat with single and paired cultures derived from single primary sporidia of forms 1 and 3 of Tilletia tritici*

Form and culture No.	Form 1				Form 3	
	205	297	299	300	209	263
Form 1:						
205	−	−	−	−	−	−
297	−	−	−	−	−	+
299	−	−	−	−	−	−
300	−	−	−	−	−	−
Form 3:						
209	−	−	−	−	−	+
263	−	+	−	−	+	−

TABLE 3.—*Results of inoculating Prelude wheat with single and paired cultures derived from single primary sporidia of forms 2 and 3 of Tilletia tritici*

Form and culture No.	Form 2				Form 3	
	288	290	291	293	209	263
Form 2:						
288	−	−	−	−	+	−
290	−	−	−	−	−	−
291	−	−	−	−	−	−
293	−	−	−	−	+	−
Form 3:						
209	+	−	−	+	−	+
263	−	−	−	−	+	−

HYBRIDIZATION OF PHYSIOLOGIC FORMS OF TILLETIA LEVIS

The tests of the pathogenicity of single and paired monosporidial cultures of *Tilletia levis* were not so extensive as those of *T. tritici*. A single test was made in which four cultures of form 5 and four of form 7 were paired in all possible combinations. The results are given in Table 4.

TABLE 4.—*Results of inoculating Prelude wheat with single and paired cultures derived from single primary sporidia of forms 5 and 7 of Tilletia levis*

Form and culture No.	Form 5				Form 7			
	219	245	264	281	223	283	286	308
Form 5:								
219	−	−	−	−	+	−	+	−
245	−	−	−	−	−	−	−	−
264	−	−	−	−	−	−	−	−
281	−	−	−	−	−	+	−	−
Form 7:								
223	+	−	−	−	−	−	−	+
283	−	−	−	+	−	−	−	−
286	+	−	−	−	−	−	−	+
308	−	−	−	−	+	−	+	−

This test showed that the infection phenomenon found for *Tilletia tritici* was also true for *T. levis*. The species apparently is hetero-

thallic, as none of the monosporidial cultures alone was able to cause infection. A number of sex groups appear to be involved. None of the cultures of form 5, when paired with one another, caused infection, but they hybridized with sexually compatible cultures of form 7. Four of the eight cultures tested belonged to one sexually compatible group. Cultures 223 and 286 of form 7 produced infection when paired with cultures 308 of form 7 and 219 of form 5. Culture 283 of form 7 caused infection only when mated with culture 281 of form 5, showing the existence of another sexually compatible group. Cultures 245 and 264 did not cause infection when paired with any of the cultures, thus indicating the existence of another sex group.

HYBRIDIZATION OF TILLETIA TRITICI AND T. LEVIS

The question of hybridization of *Tilletia tritici* and *T. levis* in nature has frequently been raised. The writer has examined over 10,000 bunted heads of wheat and has found that the rough-spored and smooth-spored forms rarely occur together in the same smut ball or even in the same head. However, all degrees of reticulation have been observed. Some of the spores were so finely reticulated that they could be distinguished only with difficulty from the nonreticulated species, while others were so coarsely reticulated that they appeared almost spiny. That there is abundant opportunity for hybridization to occur in nature is shown in a survey made by the writer. In 1930, samples of 50 to 100 smutted heads from each field were collected from fields widely separated in Washington, Oregon, and Idaho, and each head was examined for the presence of the smooth-spored and rough-spored species. In Washington 46 out of 65 collections were mixtures of *T. tritici* and *T. levis;* in Oregon 26 out of 45 were mixtures; and in Idaho 9 out of 10 were mixtures.

The results obtained by inoculating seedlings of Prelude wheat with 5 monosporidial cultures of form 3 of *Tilletia tritici*, and with 5 cultures of form 7 of *T. levis*, in all possible paired combinations, are presented in Table 5. These data show that the two species hybridize readily. Of the 10 cultures tested, 8 appear to belong to one sexually compatible group. Culture 308 of *T. levis* and cultures 263, 303, and 306 of *T. tritici* belong to one sex, while cultures 223, 285, and 286 of *T. levis* and 209 of *T. tritici* belong to the other. Although the pairing 285 + 263 did not cause infection, it is probable, in consideration of the limited number of plants used and because of similarity in reaction of these cultures to the others in all the other cases, that other factors than sex were involved. Cultures 283 and 302 did not produce infected plants and may have belonged to a sex group the complement of which was not included in this test.

The spores produced in heads infected by pairing a monosporidial culture of the reticulate-walled *Tilletia tritici* with one of the smooth-walled *T. levis* were identical in appearance with those of the latter. In this test nine interspecific crosses caused infection and the spores were invariably smooth walled and somewhat ellipsoidal in shape.

TABLE 5.—*Results of inoculating Prelude wheat with single and paired cultures derived from single primary sporidia of form 3 of Tilletia tritici and form 7 of T. levis*

Inoculum and culture No.	T. tritici, form 3					T. levis, form 7				
	209	263	302	303	306	223	283	285	286	308
T. tritici, form 3:										
209	−	+	−	+	+	−	−	−	−	+
263	+	−	−	−	−	+	−	−	+	−
302	−	−	−	−	−	−	−	−	−	−
303	+	−	−	−	−	+	−	+	+	−
306	+	−	−	−	−	+	−	+	+	−
T. levis, form 7:										
223	−	+	−	+	+	−	−	−	−	+
283	−	−	−	−	−	−	−	−	−	−
285	−	−	−	+	+	−	−	−	−	+
286	−	+	−	+	+	−	−	−	−	+
308	+	−	−	−	−	+	−	+	+	−

DISCUSSION AND CONCLUSIONS

The pathogenicity of 20 cultures of *Tilletia tritici* and 9 cultures of *T. levis*, which had been derived from single primary sporidia, was tested. In this group were three physiologic forms of *T. tritici* and two of *T. levis*. These forms had been purified by varietal straining and were pathogenically distinct. Prelude wheat inoculated with single cultures was not smutted, but the pairing of two sexually compatible cultures caused normal infection.

The problem of the interrelationship of sex and pathogenicity appears to be complicated by the existence of a number of sex groups. For example, cultures A+B may cause infection, as may C+D, but any combination of A or B with C and D will not. The existence of still other sex groups is indicated by cultures that failed to cause infection when paired with A, B, C, or D. The members of the sex groups are specific, for in no instance was a member of one group able to cause infection when paired with one of another group.

Although the sex groups were very distinct, membership within a sex group was not confined to a particular physiologic form or even to the respective species. Into one sexually compatible group fell 8 lines of form 3, 2 of form 2, and 1 of form 1 of *Tilletia tritici*, and 1 of form 5 and 4 of form 7 of *T. levis*. Form 3 of *T. tritici* readily hybridized with forms 1 and 2 of the same species and with form 7 of *T. levis*. Forms 5 and 7 of *T. levis* also hybridized. These were the only combinations of forms tested, but it is probable that hybridization between other combinations may occur and may be an important factor in the origin of new physiologic forms.

In a study of the importance of hybridization and mutation in the origin of pathogenically different physiologic forms of *Tilletia tritici* and *T. levis*, it is essential to obtain as pure a line as possible of the diploid or pathogenic phase.

Cytological studies of *Tilletia tritici* have been made by Rawitscher (*14*), Paravicini (*13*), and Dastur (*3*). These investigators found that a nuclear fusion precedes chlamydospore formation. Therefore, the chlamydospores are hybrids unless the sporidia or monosporidial cultures that unite prior to chlamydospore formation are genetically identical except for sex.

Until the present time the only method that has been used for the purification of collections, in the determination of physiologic forms, is varietal straining. This has been done by inoculating differential varieties on the theory that those forms in the collection to which the particular variety is resistant will be strained out. Although Gaines's[5] results appear to bear out this theory, this method gives no assurance that a number of physiologic forms to which the variety may be susceptible are not present. Theoretically the sporidia or gametes of a physiologic form should be genetically identical except for sex.

The cultures derived from the single primary sporidia of *Tilletia tritici* and *T. levis* are heterothallic. Consequently these cultures are haploid and functionally gametic. It should be possible to develop pathogenically pure diploid lines by mating haploid cultures of the progeny to each of their parents and in turn mating haploid cultures of the progeny of this mating to each of the original parental cultures. A repetition of this process should develop pure lines with greater certainty than the methods now used for developing pure lines of animals and the higher plants, for instead of having two variable parental gametes, one gamete would be constant.

SUMMARY

Tilletia tritici and *T. levis* are heterothallic. Wheat seedlings inoculated with individual cultures derived from single primary sporidia remained healthy, but when inoculated with paired cultures of opposite sex they produced smutted heads.

The monosporidial cultures or lines belong to a number of sex groups. The members of each group are specific in their action, as in no instance was a member of one sexually compatible group able to cause infection when paired with a member of another such group.

Wheat seedlings inoculated with a monosporidial culture of *T. tritici* paired with one of *T. levis* of opposite sex were smutted.

Monosporidial cultures of three forms of *T. tritici* and two forms of *T. levis* were found to belong to the same sex group. The spores produced by this species cross were identical in appearance with those of *T. levis*. The epispore wall was smooth, and the spores were somewhat ellipsoidal and slightly angular in shape.

Ample opportunity for hybridization occurs in nature.

By properly pairing the monosporidial cultures it should be possible to develop pathogenically pure lines of *T. tritici* and *T. levis*.

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THE VITAMIN A, B, C, AND G CONTENT OF SULTANINA (THOMPSON SEEDLESS) AND MALAGA GRAPES AND TWO BRANDS OF COMMERCIAL GRAPE JUICE [1]

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INTRODUCTION

During recent years a large annual surplus of grapes has been produced in the United States, and the development of new grape products to take care of this increasing supply is at present an important phase of the grape industry. Because of the general interest in a fruit so abundantly grown, it seemed desirable to study the nutritive value of grapes and grape juices more extensively than had previously been done. Raisins have received the attention of several investigators (*3*, *12*),[2] who apparently agree that this product contains very little if any vitamin A, a small amount of vitamin B, and no vitamin C. However, up to the present time very few data have been published on the vitamin content of fresh grapes and their juices, and because of the value of such information the present experiments were planned to determine the amount of vitamins in two varieties of fresh grapes as well as in two brands of commercial grape juice.

Sultanina (Thompson Seedless) and Malaga (*Vitis vinifera*), European varieties, were the grapes selected for study. The Sultanina or Thompson Seedless is a raisin grape constituting about 90 per cent of the total crop of seedless grapes. In this country it is grown principally in California. The Malaga, a table grape, is also used for raisins and, together with the muscat, forms the chief source of the seed or seeded raisins.

In addition to the fresh grapes, two brands of commercial grape juice were analyzed for their vitamin content. The first, designated as commercial juice No. 1, was a mixture of juices approximately one-third from the Flame Tokay and two-thirds from the Zinfandel, European table and juice grapes, classified as *Vitis vinifera*. In the commercial process the juices after extraction from the fresh fruit are filtered and placed in cold storage until needed. Upon removal from cold storage, they are refiltered, sterilized through a machine at a temperature not to exceed 155° F., and bottled. The bottles of juice are then kept in a water bath held at 150° for 45 minutes. Sometimes a slight amount of tartaric acid is found necessary to bring the composition of the product to the standard formula used by the company. No other ingredients are added to the juice.

Commercial juice No. 2 was prepared from Concord grapes (*Vitis labrusca*), an American variety. In preparing this juice the fruit is washed, stemmed, and crushed, heated to about 135° F., and the juice pressed out. The juice is pasteurized and stored in 5-gallon glass

[1] Received for publication June 10, 1931; issued February, 1932. Presented before the division of medicinal chemistry of the American Chemical Society, at Indianapolis, Ind., Apr. 1, 1931.

[2] Reference is made by number (italic) to Literature Cited, p. 69.

carboys for several weeks to a few months to permit settling; it is then siphoned into bottles and again pasteurized. Sugar is generally an added ingredient.

VITAMIN A

In the present study the vitamin A content of the grapes and grape juices was determined by the Sherman and Munsell method (*14*). The basal diet was irradiated to supply vitamin D. After the customary depletion period of from four to five weeks, certain groups of rats were fed daily six times per week weighed portions (1, 3, and 5 gm., respectively) of seeded Malaga grapes. Certain other groups received the Sultanina in the same amounts at the same intervals. The commercial juices were given in daily doses of 2, 3, and 5 c. c. for the same number of days each week. The results are shown in Figure 1 and Table 1.

TABLE 1.—*Survival of rats receiving various quantities of grape juice as the sole source of vitamin A*

Test food	Daily portion, 6 times per week	Rats	Average weight of rats at 4 weeks of age	Average weight of rats at end of fore period	Average time of fore period	Average time of survival after fore period	Average total time of survival after 4 weeks
	C. c.	*Number*	*Grams*	*Grams*	*Days*	*Days*	*Days*
Commercial grape juice No. 1[a]	2	6	45.8	95.2	33.6	21.7	55.3
	3	8	45.9	99.9	33.9	[b] 23.3	57.1
	5	8	43.4	95.0	33.5	27.2	60.7
	0	7	46.1	99.3	34.3	30.3	64.6
Commercial grape juice No. 2 [c]	2	8	56.0	108.2	35.8	23.6	59.4
	3	8	52.5	101.2	35.6	19.5	55.1
	5	8	50.2	98.9	35.9	23.6	59.5
	0	8	52.9	105.8	35.8	16.3	52.1

[a] A mixture of juices, one-third from the Flame Tokay and two-thirds from the Zinfandel, which are European table and juice grapes classified as *Vitis vinifera*.
[b] 1 rat lived out the full experimental period.
[c] Juice prepared from Concord grapes, *V. labrusca*.

Sherman (*12*) reported the presence of vitamin A both in grapes and in grape juice, but the variety of grapes was not indicated. He stated that an ounce of grapes contained 16 to 22 units of vitamin, i. e., 0.57 to 0.7 unit per gram. If a unit of vitamin is present in that amount of the test food necessary to produce a gain of 25 gm. in eight weeks, then 1.75 to 1.43 gm. of the fruit were necessary to produce this unit gain. In the present study, in order to obtain a gain of 25 gm. in eight weeks, it was necessary to feed approximately 5 gm. of grapes in the cases of both Sultanina and Malaga. These results indicate that Malaga and Sultanina grapes are less rich in vitamin A than the grapes studied by Sherman. Nevertheless, the growth response induced by these varieties shows the presence of a small but measurable quantity of vitamin A.

Neither of the commercial juices tested showed any indication of the presence of vitamin A. With but one exception the animals died long before the termination of the experiment and showed no better growth than the negative controls. (Table 1.) Upon autopsy the gross anatomical changes due to a deficiency of vitamin A were found to be as severe in the test animals as in the controls.

VITAMIN B (ANTINEURITIC)

Recorded data furnish but scanty information regarding the vitamin B content either of grapes or of their juices. Osborne and Mendel in 1920 (*10*) reported that when 10 c. c. of commercial grape juice was fed daily it was found to contain some "water soluble B" but in amounts insufficient for the normal growth of rats. Sherman (*12*) reported the occurrence of 7 to 9 units of vitamin B per ounce of grapes. Both of these investigators, however, were considering the vitamin B complex before cognizance had been taken of the antipellagric factor. There appears to be no recent data regarding the vitamin B content of these products.

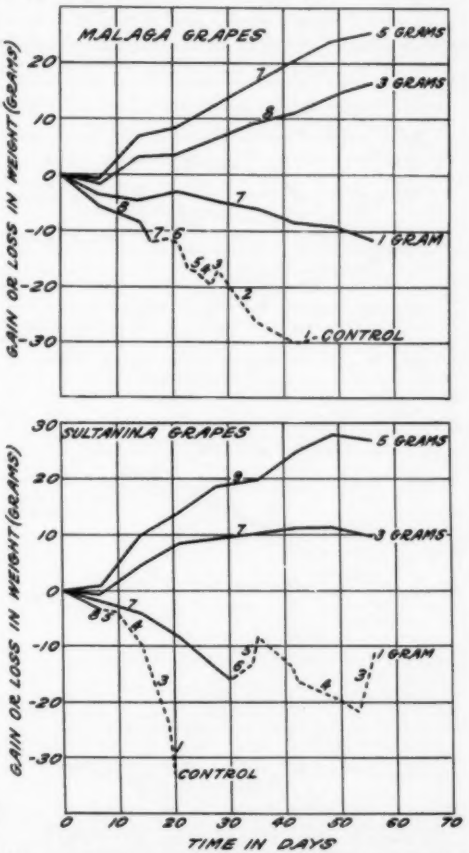

FIGURE 1.—Average gain or loss in weight of groups of young rats fed Sultanina and Malaga grapes (*Vitis vinifera*) as the sole source of vitamin A. The broken lines begin at a point indicating the occurrence of the first death in the group. The numerals along each curve show the number of rats surviving at all times during the test period. The weighed quantity of grapes fed daily six times a week is indicated at the end of the curves

The vitamin B (antineuritic) tests were carried out according to a method worked out in this laboratory, similar to that outlined by Chase (*1*). Rats, 28 days old, were placed on diet 107 G, which furnished, with the exception of the antineuritic vitamin, all factors necessary for normal growth and apparent well-being of the animals. This diet had the following composition: Vitamin B-free casein, 18 per cent; starch, 58 per cent; yeast (autoclaved four hours at 20 pounds pressure), 10 per cent; Osborne and Mendel salt mixture, 4 per cent; butterfat, 8 per cent; and cod-liver oil, 2 per cent. All of the animals were kept on this vitamin B-free diet for two weeks, a period of time judged from former observations in this laboratory to be sufficient to deplete the animals of their store of the antineuritic vitamin.

At the end of this depletion period the rats were given weighed or measured portions of the material to be tested. Both varieties of grapes were readily consumed, but a temporary difficulty was expe-

rienced in getting some of the animals to take the grape juice. The Malaga grapes, after the removal of the seeds, were fed in portions of 0.5, 1, 2, 3, 5, and 6 gm., respectively, while the Sultanina grapes were given in amounts of 3, 5, and 6 gm. Each of the two samples of juice were fed daily from small glass containers in 2, 3, and 5 c. c. portions. The results are summarized in Figures 2 and 3.

Figure 2 shows that 5 and 6 gm. of fruit in the case of both Malaga and Sultanina grapes induced approximately the same gain in weight, indicating that the maximum effect of the grapes as a source of vitamin B had been reached. A maximum average gain in weight of 17.5 gm. resulted from feeding both 5 and 6 gm. of Malagas, while 5 gm. of Sultanina produced an average gain of 18.8 gm. and 6 gm. portions of this same grape gave 21.6 gm. gain in weight. These rates of growth of the test animals indicate that the two varieties of grapes are fair sources of vitamin B.

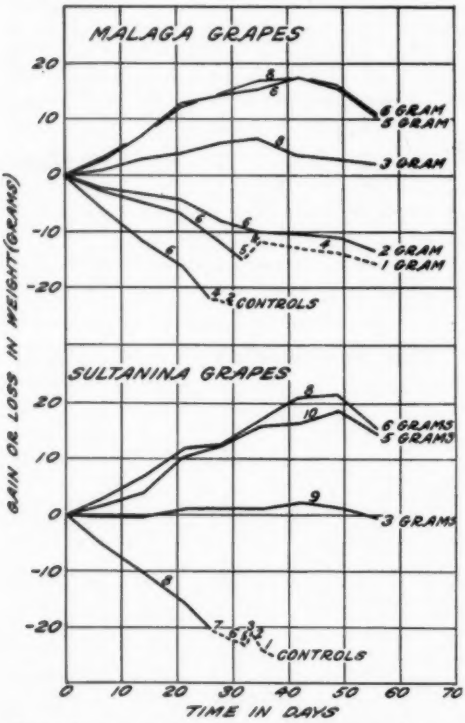

FIGURE 2.—Average gain or loss in weight of groups of young rats fed Sultanina and Malaga grapes (*Vitis vinifera*) as the sole source of vitamin B (antineuritic). The broken lines begin at a point indicating the occurrence of the first death in the group. The numerals along each curve show the number of rats surviving at all times during the test period. The weighed quantity of grapes fed daily six times a week is indicated at the end of the curves

From Figure 2 it may also be observed that the growth curves for these animals show a maximum point between the sixth and seventh week of the test, after which there is a loss in weight to the end of the period. It was difficult to obtain as good grapes at the end of the season as had been fed through the major part of the experiment, but this consistent drop in weight in all of the animals can not be explained satisfactorily on the basis of poor-quality grapes, since only a comparatively few animals received the inferior product. It is entirely possible that another factor necessary for normal growth was absent from the diet, and upon the depletion of the reserve store of this factor in the animal body the growth curves began to show a decline.

The failure of commercial juice No. 1, in 2, 3, and 5 c. c. daily portions, to induce growth indicates the absence of any measurable quantity of vitamin B in this grape juice. (Fig. 3.) On the other hand,

Figure 3 shows that commercial juice No. 2 contains the antineuritic vitamin. The amount is only minimal, however, since a daily portion of 5 c. c. of this juice induced a total gain in weight of only 3 to 4 gm. during the entire test period.

VITAMIN C

The vitamin C content of grapes and grape juices appears to have been more extensively studied than that of any of the other vitamins. Chick and Rhodes (*2*) found the juice of grapes to be about one-tenth as rich in the antiscorbutic vitamin as oranges. Givens and Macy (*5*) found no antiscorbutic properties in dehydrated grape juice which was 14 to 20 months old at the time of testing. According to Merjanian (*9*), grapes contain vitamin C, the amount varying with the kind of grapes and their freshness. Taking orange juice as 100 for a standard of comparison, Sherman (*12*) reported that grapes and grape juice have a potency of 4 to 5.

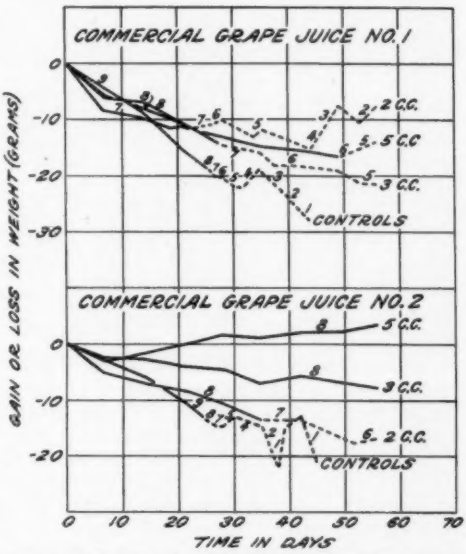

FIGURE 3.—Average gain or loss in weight of groups of young rats fed commercial grape juices (No. 1, juice from Flame Tokay and Zinfandel varieties, *Vitis vinifera;* No. 2, juice from Concord grapes, *V. labrusca*) as the sole source of vitamin B (antineuritic). The broken lines begin at a point indicating the occurrence of the first death in the group. The numerals along each curve show the number of rats surviving at all times during the test period. The measured quantity of juice fed daily six times a week is indicated at the end of the curves

The vitamin C tests on commercial grape juice No. 1 were carried out after the method of Sherman, LaMer, and Campbell (*13*), the 90-day test period being used. The basal diet designated as 12 D was a modification of that used by Sherman and had the following composition: Equal parts mixture of bran and oats, 57 per cent; table salt, 1 per cent; butterfat, 9 per cent; heated skim-milk powder, 30 per cent; cod-liver oil, 1 per cent; and yeast, 2 per cent. Six, eight, and ten cubic centimeters of the commercial juice No. 1 were fed from a graduated pipette to groups of guinea pigs every day except Sunday during the test period. Table 2 shows that the survival period of the test animals on this brand of juice was no longer than that of the negative controls and upon autopsy they showed just as severe symptoms of scurvy. Such evidence indicates that commercial grape juice No. 1 contains no vitamin C.

TABLE 2.—*Survival of guinea pigs receiving various quantities of commercial grape juice No. 1 (juice of Flame Tokay and Zinfandel grapes, Vitis vinifera) as the sole source of vitamin C*

Daily dose, 6 times per week	Guinea pig No.	Weight at beginning	Maximum weight	Weight at end	Survival	Severity of scurvy symptoms at autopsy
		Grams	*Grams*	*Grams*	*Days*	
10 c.c.	245 F	338	371	202	35	Moderate to severe.
	254 F	320	316	203	30	Do.
	268 F	323	350	232	32	Do.
	272 F	327	336	216	29	Moderate.
8 c.c.	251 F	322	384	210	39	Do.
	252 F	325	335	206	28	Severe.
	257 F	323	363	195	32	Do.
	261 F	328	337	214	27	Moderate to severe.
	274 F	319	319	198	28	Moderate.
6 c.c.	256 F	336	401	213	36	Moderate to severe.
	263 F	329	349	182	30	Mild.
	269 F	324	341	166	33	Severe.
	273 F	310	310	242	19	Mild.
0 c.c.*	162 F	351	427	247	35	Moderate to severe.
	262 F	327	347	222	25	Severe.
	118 M	344	344	245	27	Moderate.
	122 M	409	467	267	35	Severe.
	152 M	327	379	203	36	Do.
	153 M	300	390	202	37	Do.
	170 M	402	459	285	37	Do.
	177 M	367	367	223	24	Do.
	182 M	361	413	254	35	Do.
	259 M	351	423	244	33	Do.
	270 M	316	322	217	37	Do.

* The 9 males were controls carried with other experiments in this laboratory.

Tests on the commercial juice No. 2 and the fresh grapes were conducted according to the method of Höjer (*7*, *8*), who determines the degree of scurvy by a study of the pathological condition of the teeth. Attention is focused especially on the microscopical examination of a cross section of the root of the incisor tooth of the guinea pig. Höjer claims that this method is more sensitive than the one used by Sherman. A comparative study of these methods has been made by Eddy (*4*) and Goettsch and Key (*6*). Following Höjer's technic, they found that twice the amount of test food is required to afford complete protection when the criterion depends on a histopathological examination of the teeth, than is apparently necessary when judgment is based on the gross external and internal anatomical changes. Höjer has shown that differential changes in tooth structure begin to take place between the tenth and fourteenth day of the test and are materially sharpened as the experiment continues.

In the present study an 18-day test period was adopted. During this time 10 and 12 c. c. portions of grape juice were fed to guinea pigs weighing between 300 and 350 gm. Because earlier evidence had indicated that there was little if any vitamin C present in grape juice it was considered unnecessary to feed this test food in smaller quantities. Twelve cubic centimeters was the maximum daily amount which the guinea pigs would take. Seven animals were used. Seeded Malaga grapes were given in 2, 5, 10, and 15 gm. portions to 16 animals, while Sultanina grapes in the same quantities were fed to 12. On the eighteenth day the animals were killed, the incisor teeth removed, and sections prepared.

Figure 4, A, shows the structure of a normal tooth taken from one of the control animals that had received an adequate supply of vitamin C from a daily allowance of cabbage. Figure 4, B, portrays the con-

dition of the tooth of one of the negative control animals fed only the basal diet with no antiscorbutic vitamin for 18 days. From Figure 4, A, it may be observed that the normal tooth possesses a wide band

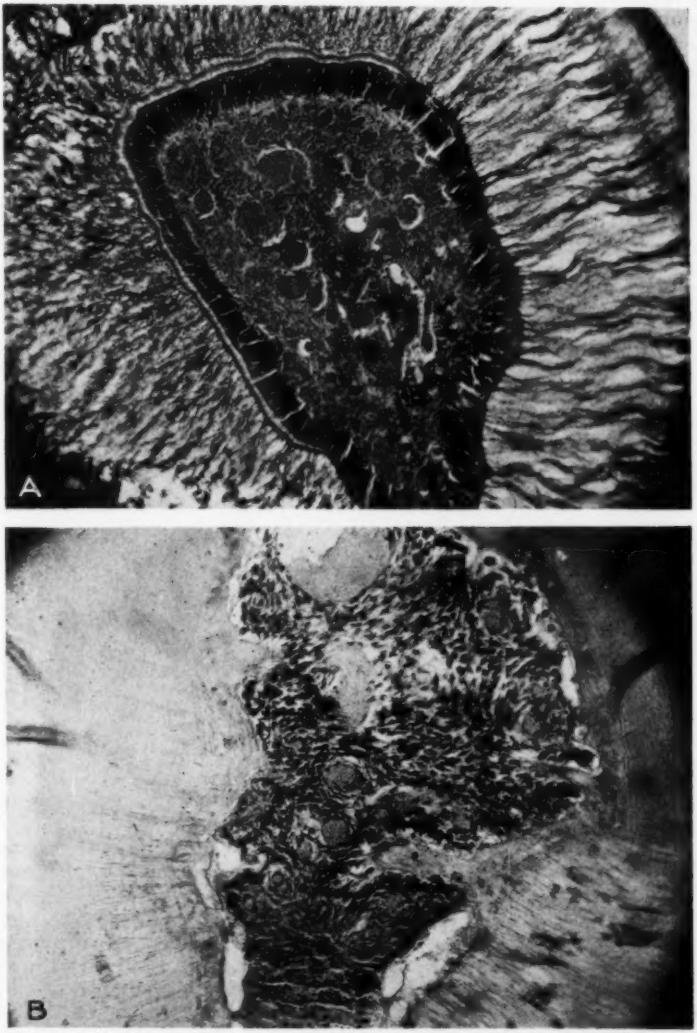

FIGURE 4.—A, Section of a normal incisor tooth taken from a guinea pig fed a diet complete in all necessary factors; B, section of a tooth from a scorbutic guinea pig that received a vitamin C deficient diet for 18 days

of evenly stained dentine inside of which is a narrow layer of uncalcified predentine, and then a row of very tall parallel columnar odontoblasts surrounding the normal pulp. An inadequate supply

of vitamin C causes the layer of dentine to become narrower, the predentine becomes calcified, and the odontoblastic layer of cells loses its soldierlike formation, while the cells themselves become shorter and gradually work their way into the pulp cavity to function as osteoblasts, the bone-forming instead of the dentine-forming cells. Consequently the scorbutic condition portrayed in the tooth structure in Figure 4, B, can be readily recognized even before the guinea pig develops outward symptoms of scurvy.

All of the teeth taken from the guinea pigs receiving commercial grape juice No. 2 showed marked pathological conditions analogous

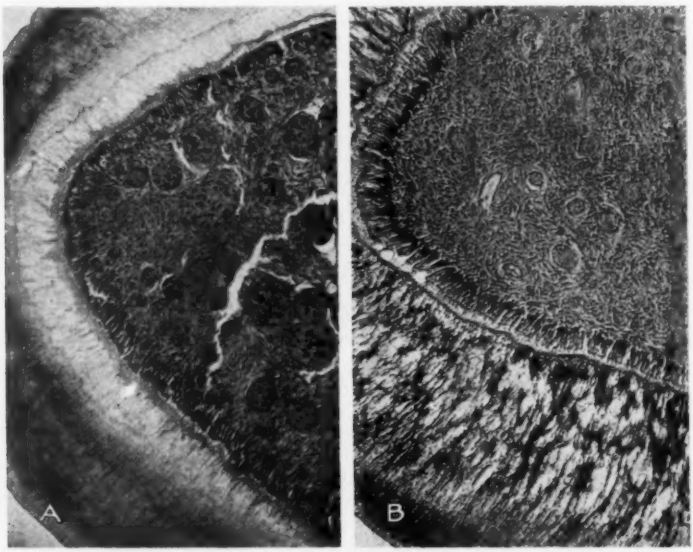

FIGURE 5.—A, Section of an incisor tooth taken from a guinea pig receiving 15 gm. of Malaga grapes as the sole source of vitamin C; B, section of a tooth from a guinea pig receiving 15 gm. of Sultanina grapes as the sole source of vitamin C

to those portrayed in Figure 4, B. Thus, it is concluded that this juice contains no appreciable amount of vitamin C.

The sections prepared from the teeth of animals fed 2-gm. and 5-gm. supplemental portions of either Malaga or Sultanina grapes showed that neither variety in these quantities prevented the occurrence of severe pathological changes in the teeth. While there was a slight protection in the teeth of guinea pigs fed 10 gm. of grapes, still this quantity furnished far too little vitamin C to give a normal tooth structure. Figure 5, A and B, representative of the teeth of those animals receiving 15 gm. daily of Malaga and Sultanina grapes, respectively, shows that even this quantity of the fruit was insufficient to afford border-line protection. Of the two varieties of fresh grapes studied, Sultanina contained the greater amount of vitamin C (fig. 5, B); 15 gm. of this fruit offered approximately the same protection as 2 c. c. of orange juice. (Fig. 6.)

VITAMIN G (B_2)

No report of any kind has been found that gives the vitamin G content of grapes or grape juice.

FIGURE 6.—Section of an incisor tooth taken from a guinea pig receiving 2 c. c. of fresh orange juice daily as the sole source of vitamin C

A method for the determination of vitamin G was worked out in this laboratory, and is similar in some respects to that used by Sandels (*11*). For a period of two weeks, 28-day-old rats were given the basal diet alone in order to deplete them of any vitamin G that might

be stored in their bodies. Vitamin B was supplied in the form of an 80 per cent by weight alcoholic extract of white corn which up to the present time has been found in this laboratory to be the most satisfactory source of vitamin B free from grossly interfering amounts of vitamin G. The basal diet consisted of the following ingredients: Purified casein, 18 per cent; Osborne and Mendel salts, 4 per cent; butterfat, 8 per cent; cod-liver oil, 2 per cent; starch, $68-X$ per cent;[3] and corn extract, X per cent. Sultanina grapes were fed to the different groups of animals in 0.5, 1, 2, 3, and 5 gm. daily portions six times per week; the Malagas were given in the same manner in 2, 4, and 6 gm. amounts, while each grape juice was fed in 2, 3, and 5 c. c. allotments. The experiment covered a period of 10 weeks, but no significant changes occurred during the ninth and tenth weeks, and therefore only 8 weeks of the test are portrayed in the curves showing the rate of growth of the animals. (Fig. 7.)

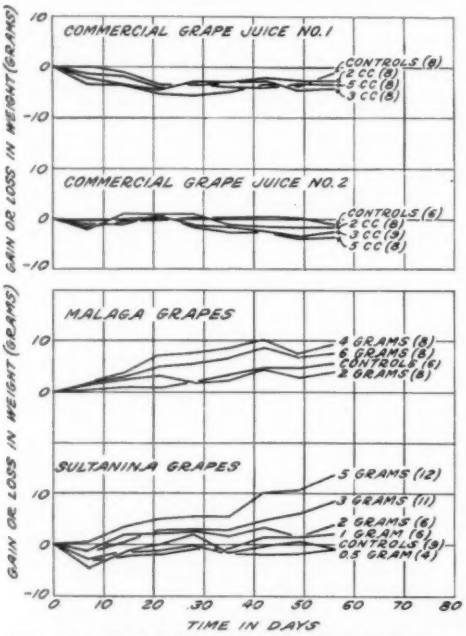

FIGURE 7.—Average gain or loss in weight of groups of young rats fed Sultanina and Malaga grapes (*Vitis vinifera*) and two brands of commercial grape juice (No. 1 from Flame Tokay and Zinfandel grapes, *V. vinifera*; No. 2 from Concord grapes, *V. labrusca*) as the sole source of vitamin G (B_2). The quantity of grapes in grams and the cubic centimeters of juice fed daily six times per week is indicated at the end of each respective curve. The numerals in parentheses indicate the number of animals subjected to each respective test

The animals that received an insufficient amount of vitamin G first showed woolliness of fur and then a thinning of the hair, especially on the sides of the head. About the sixth week of the test a brownish-red discharge appeared caked along the inner sides of the forelegs and paws, and considerable of this material appeared on the nose and whiskers of the animals. Although it strongly resembled blood, there was no sign of broken skin or bleeding on the legs directly under the deposit, and it was not possible to obtain a positive blood reaction when the benzidine test was used. Upon autopsy, the contents of the stomach and intestines appeared very similar to this discharged material. Very often lesions appeared on the side of the head out from the eyes and the corners of the mouth. The animals seemed very nervous and spent considerable time rubbing their heads. Priapism was very commonly found.

[3] X represents the amount of extract, evaporated on cornstarch, obtained through the extraction of 90 gm. of corn.

Sultanina grapes in 5-gm. daily portions induced an average gain of 13.7 gm. for the entire eight weeks and therefore contain a small but significant amount of vitamin G. The animals receiving the Malagas did not make sufficient growth, as shown in Figure 7, to indicate the presence of any of this vitamin. From the same figure it may be seen that neither juice contains any vitamin G.

SUMMARY

Fresh Sultanina (Thompson Seedless) and Malaga grapes (*Vitis vinifera*) and two brands of commercial grape juice (No. 1, a mixture of juices from Flame Tokay and Zinfandel varieties, *V. vinifera*, and No. 2, the juice from Concord grapes, *V. labrusca*) were tested for their vitamin A, B (B_1), C, and G (B_2) content. The results showed that:

Both varieties of grapes contained a small but measurable amount of vitamin A. There was no evidence of this vitamin in either juice.

Vitamin B (antineuritic) was present in fair amounts in both kinds of fresh grapes tested and in small quantity in the commercial juice designated as No. 2. Commercial juice No. 1 did not contain vitamin B in a measurable quantity.

Fifteen grams of fresh grapes fed daily were found to contain insufficient amounts of vitamin C to protect guinea pigs from scurvy as determined by the Höjer method. This quantity of Sultanina grapes furnished approximately the same protection as 2 c. c. of orange juice and contained more of the antiscorbutic vitamin than the Malaga grapes. There was no indication of vitamin C, as determined by the Sherman method, in commercial juice No. 1. Tests made by the Höjer method indicated the absence of antiscorbutic vitamin in commercial juice No. 2.

Sultanina grapes appeared to contain a minimal amount of vitamin G, while Malaga grapes and both juices were lacking in this vitamin.

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FACTORS INFLUENCING THE CHANGES IN OXIDATION-REDUCTION POTENTIAL ON THE REDUCTION OF METHYLENE BLUE IN MILK[1]

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INTRODUCTION

The methylene blue reduction test as it is used to-day is one of the most practical tests for determining the quality of milk. Although the early conceptions of biological reduction processes have been completely reorganized, the selection of the dye and the concentration employed have not been changed by a more fundamental understanding of the factors involved. The newer conception of the methylene blue reduction test is the result of studies of reducing intensities of biological systems. For the most part, these studies have been of a more fundamental and theoretical nature, with less emphasis upon their practical application to the reductase test.

In this paper an attempt is made to correlate the factors influencing the changes in oxidation-reduction potential with the reduction of methylene blue in milk.

LITERATURE REVIEW

Fred (*15*)[2] presented an excellent historical review of the early literature pertaining to dye reduction by microorganisms. As a result of his own researches, Fred firmly established the dependence of reduction time of methylene blue in milk on the quantitative and qualitative aspects of the original bacterial flora. The probability that the reduction of methylene blue might be due to some constituent of the milk was suggested by Barthel (*1*) and Hastings (*18*).

The first evidence that the reducing intensity of bacterial cultures might be measurable in terms of electrode potential was presented by Gillespie (*16*). In measuring the reduction potentials of bacterial suspensions and of water-logged soils, he observed a trend toward more negative reducing intensities.

Clark (*5*) measured the equilibrium potentials of the systems methylene blue—methylene white and indigo-indigo white. As a result of these studies, he established quantitative values for the different reducing intensities indicated by these systems.

Following a study of the significance of anaerobiosis, Hall (*17*) states that adsorption plays an important rôle in the decolorization of dyes by porous substances such as animal and plant tissues.

[1] Received for publication July 13, 1931; issued February, 1932. Contribution No. 135, Department of Bacteriology, Kansas Agricultural Experiment Station.

[2] Reference is made by number (italic) to Literature Cited, p. 82.

Hastings, Davenport, and Wright (*19*) conclude that the reduction of methylene blue is very intimately connected with the vital processes of the cell rather than with any extracellular by-products.

By microinjection of several reversible oxidation-reduction indicators into *Amoeba proteus* and *A. dubia*, Needham and Needham (*22*) and Cohen, Chambers, and Reznikoff (*8*) found that these protozoa maintain a fairly constant reduction potential.

In 1920 Clark (*5*) presented a comprehensive basis for interpreting, in terms of electrode potential, the results given by biological reduction of reversible oxidation-reduction systems. Clark and his associates (*6*) made a quantitative study of the potential of a large number of the oxidation-reduction indicators, including methylene blue, and determined the relative position of these indicators on the potential scale. They presented the time : potential curves of samples of inoculated, bottled, and fresh milk.

By measuring the potentials of cells, extracts, and cultures, Cannan, Cohen, and Clark (*3*) showed a general correlation between the reduction potential of a cell suspension, the cellular reduction of a dye, and the reduction potential of the same dye as determined in pure solution. They showed also that different species of bacteria attain different levels of reducing intensity and follow different courses.

Coulter (*9*) observed the parallelism between the reducing intensities induced by bacterial respiration and those attained by the removal of oxygen from sterile bouillon. He concluded that the development of the characteristic negative limits of intensity in bacterial cultures can not be attributed entirely to reductive processes directly dependent upon the action of living cells.

Cohen (*7, p. 16–17*) states:

> Bacterial cultures in broth and synthetic media develop progressively increasing reducing intensities which have been followed electrometrically. Oxidation-reduction indicators, within the limits imposed by chemical reactivity and narrow useful range confirm the time : potential curves. The levels of reduction potentials attained by cultures of different bacteria are more or less different and characteristic.

Sterile broth when protected from the atmosphere by a vaseline seal is capable of reducing a number of dyes, including methylene blue, as demonstrated by Dubos (*10*).

Thornton and Hastings (*24*) observed a very close similarity between the potential : time curves of milk with and without methylene blue. Although the potentials of the zone of visible reduction of methylene blue in milk were found to be variable, they were always more positive than the theoretical zone in pure solutions of this dye at the same pH value. These authors were able to decolorize the dye in milk by deaeration and to restore the blue color by aeration. They state (*25*) that their work tends to confirm Barthel's (*2*) theory of methylene blue reduction in milk.

It was shown by Fildes (*11*) that the period required for the germination of spores of *Bacillus tetani* depends mainly on the time required for the medium to reach a suitable reducing intensity. The same writer (*12*) reported that the subcutaneous tissues of a living guinea pig maintain an E_h on the positive side of reduced methylene blue, and that the E_h becomes more negative at the death of the animal.

Lepper and Martin (*21*) reported that cooked-meat media when exposed to air was reduced by cultures of two aerobes and five ana-

erobes. Hewitt (*20*) measured the potentials of three cultures in several kinds of medium, and found that *Corynebacterium diphtheriae* and *Staphylococcus aureus* were usually able to attain more negative reducing intensities than a hemolytic streptococcus.

METHODS

Burnished platinum foil electrodes were chosen after an extensive comparison of results obtained in parallel tests with gold foil, gold wire, platinum wire, and gold-plated platinum electrodes. Electrodes 1 cm. square were submerged to a depth of about 2 inches in approximately 50 c. c. samples. By means of suitable switches leads from six electrodes were connected to a Leeds and Northrup type K potentiometer. A saturated KCl calomel half cell was used as the reference electrode. Connections were made from the reference electrode to the

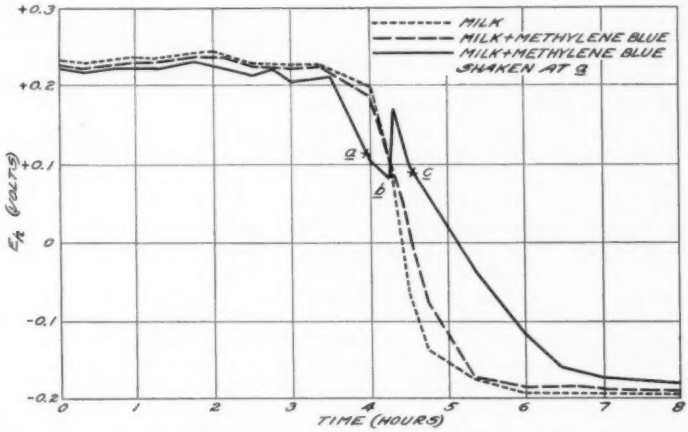

FIGURE 1.—Potential : time curves of a sample of market milk with and without the addition of methylene blue

samples under measurement by means of a saturated KCl liuqid junction and saturated KCl=agar bridges. Samples were incubated in a water bath maintained at 37° ± 1° C. Potential readings taken at suitable intervals were reduced to the hydrogen standard (E_h) and plotted as the ordinate against time as the abscissa.

EXPERIMENTAL DATA

EFFECT OF THE PRESENCE OF METHYLENE BLUE IN MILK ON THE FORM OF THE POTENTIAL : TIME CURVES

A sample of market milk was divided into three parts, to two of which was added the standard amount of methylene blue ordinarily employed in the reductase test (1: 200,000). The oxidation-reduction potentials of these three identical samples were followed through the entire course of the reduction process. The marked similarity of the potential: time curves (fig. 1) of the samples with and without methylene blue suggests that this concentration of dye has no marked effect upon the trend of the potential drift.

The effect of incorporating oxygen by shaking is illustrated in Figure 1 by the solid line. The blue color had completely disappeared at point *a*, and at point *b* the sample was shaken vigorously for 30 seconds. The return of the potential to approximately the original positive values was accompanied by a return of the blue color. The color had again disappeared at point *c*. The second drop in potential in this sample was not so rapid as that occurring in the other two samples, probably because of the deterring effect of the incorporated oxygen on potential drift. Attention is called to the marked similarity of the curves in Figure 1 with those published by Thornton and Hastings (*24*) illustrating a similar experiment. The form of the potential : time curves, the zones of decolorization, the effect of incorporated oxygen, and the negative limits attained are almost identical with the results obtained by Thorton and Hastings.

In further studies, air was bubbled into a sample of milk plus methylene blue after the potential had reached the negative E_h limit of -0.2 volt. The potential returned almost to the positive extreme, but the blue color did not return. The potential was observed 30 minutes after the positive extreme had been reached and was found to be falling rapidly to the negative side.

EFFECT OF THE BACTERIAL FLORA IN MILK ON THE FORM OF THE POTENTIAL TIME : CURVES

Clark and his associates (*6*) and Frazier and Whittier (*13, 14*) reported that various species of bacteria run characteristic courses and attain different levels of reducing intensity, thus giving rise to various though characteristic forms of potential:time curves. The results of these investigators suggest a plausible explanation for some of the difficulties commonly encountered in the practical application of the methylene blue reduction test. Frequently, the time elapsing between the first evidence of diminution of color and complete decolorization of the dye is so prolonged as to render the end point very indistinct. It is not uncommon to find samples of milk in which 30 to 60 minutes elapse between the beginning and the end of visible reduction of methylene blue. Apparently this is due to the type or types of organisms which dominate the flora of the milk.

In order to determine whether the variations reported in the literature on pure culture studies could be reproduced with the mixed flora of market milk, the oxidation-reduction potentials of samples incubated at various temperatures were noted. It was commonly observed that most fresh milk gave a potential : time curve which fell rapidly through the zone of reduction of methylene blue in less than five minutes, whereas for the same milk after 48 hours at 3° to 5° C. the time elapsing between the beginning and the end of visible reduction frequently exceeded 30 minutes. Although the trend of the curves obtained from samples incubated at higher temperatures were quite variable, the results emphasize the significance of the dominating organisms in the flora as a factor which may be responsible for the slow reduction of the dye in some samples of milk. Plotting the potential drift of a large number of samples of milk has shown considerable variation in the form of these curves. The curve for a sample of fresh milk is characterized by a rapid fall from the positive to the negative extremes. If a sample of milk

giving rise to this form of curve contains the standard amount of methylene blue, the interval between the beginning and the end of visible reduction will be short, usually less than five minutes. It was commonly observed that the bacterial flora of milk held 48 hours at 3° to 5° C. gave a potential : time curve which fell slowly to the negative extreme. This was accompanied by a slow decolorization of the methylene blue, frequently observed to extend over a period of 30 minutes. It is evident that a rapidly falling potential will pass through the zone of visible reduction in less time than one that falls slowly, thus explaining the variations in time required for decolorization of the dye in different samples of milk.

EFFECT OF FAT ON THE ZONE OF REDUCTION OF METHYLENE BLUE

It was noted that when the standard amount of methylene blue was added to skim milk the dye decolorized between the E_h values of zero and +0.05 volt. The potentials of this zone are approximately 0.1 volt more negative than the zone of decolorization of the same amount of methylene blue when added to whole milk. It was also observed that the same amount of methylene blue, when added to cream, decolorized between the E_h limits of +0.3 and +0.2 volt. The potential of this zone is approximately 0.1 volt more positive than that observed for whole milk.

To determine more definitely the potential of the zone of reduction of methylene blue in milk of various percentages of fat, sterile 40 per cent cream and sterile skim milk were mixed in suitable proportions to obtain six solutions containing 40, 30, 20, 10, 5, and 0 per cent of fat. The solutions were inoculated equally with a 24-hour culture of *Streptococcus lactis*, and the standard amount of methylene blue was added to each. The oxidation-reduction potentials were followed, and the potential : time curves of the six solutions are presented in Figure 2. The potentials of the zone of reduction of the methylene blue are indicated by triangles at the right of the respective graphs.

The potentials of the zone of reduction of methylene blue in skim milk are more negative than those observed in the case of cream. Methylene blue was reduced in skim milk between the E_h values of +0.092 and +0.050, and in cream between the values of +0.275 and +0.245. The zones of reduction in the other samples, without exception, became more positive as the percentage of fat was increased. The potentials of the zone of reduction of methylene blue in skim milk approximate more closely the theoretical zone for this dye in aqueous solution as reported by Clark and his associates (*6*).

The potentials of more than 25 samples of skim milk have been measured, and in no case has the methylene blue been observed to be reduced at a potential more positive than +0.1 volt. The zone of reduction of methylene blue in 50 samples of 40 per cent cream was never observed to be more negative than +0.225 nor more positive than +0.3 volt. It may be noted that the form of the potential: time curves is not affected by varying the percentage of fat.

Other factors being equal, it would require a somewhat longer time to reduce methylene blue in skim milk than in 40 per cent cream with the same original bacterial content. In the case of skim milk the oxidation-reduction potential must be carried to the negative

limits of approximately +0.05 volt, whereas in the case of 40 per cent cream visible reduction is usually complete at an E_h of +0.25 volt.

The exact manner in which fat alters the zone of reduction is not known. Studies of oxidation-reduction phenomena have been limited largely to simple equilibria in aqueous solutions. Many of the fundamental aspects of the simplest systems are yet to be understood. The present status of our knowledge of these simple equilibria certainly does not encourage speculations with respect to complex systems of unknown composition, as is the case with biological fluids. The more positive zone of reduction of methylene blue in cream than in skim milk may involve some unknown factors in oxidation-reduction equilibria. The work of Hall (*17*) suggests the possibility that adsorption of the dye may play a rôle in this connection.

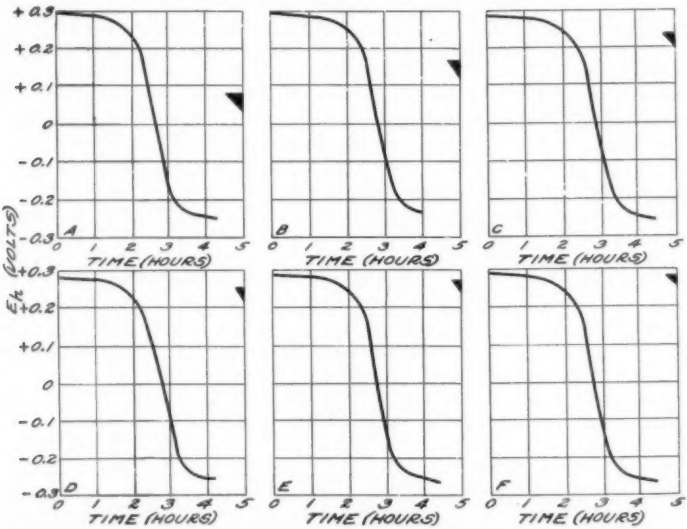

FIGURE 2.—Relation between the percentage of fat in milk and the zone of reduction of methylene blue: A, Skim milk; B, 5 per cent fat; C, 10 per cent fat; D, 20 per cent fat; E, 30 per cent fat; F, 40 per cent fat. Potentials of the methylene blue zone of reduction are indicated by black triangles

Cursory experiments indicate that approximately four times as much methylene blue must be added to 40 per cent cream to give the first perceptible tinge of blue as is required for skim milk. Conversely, in the decolorization of the dye, the point at which color is no longer discernible will be reached sooner in cream than in skim milk. The addition of the standard amount of methylene blue imparts a distinct blue color to skim milk, but only a very faint blue tinge to 40 per cent cream. The loss of only a slight amount of the blue dye by reduction in cream results in a disappearance of color. Obviously, this point of visible reduction will be reached very soon after the potential begins its swing toward more negative values. In the case of skim milk the blue color persists until more negative potentials have left only a relatively small percentage of the dye in the oxidized or blue form. Although this is not offered as a complete

explanation of the more positive zone of reduction of methylene blue in cream, the quantity of dye necessary for colorization or decolorization must be taken into consideration.

If the detection of color is dependent on a requisite minimum number of molecules of the oxidized form of the dye, one would expect the addition of larger amounts of methylene blue to lower the zone for cream to the approximate E_h values observed for skim milk. The results of experiments in which various amounts of dye were added are presented in Figure 3.

EFFECT OF THE CONCENTRATION OF DYE ON OXIDATION-REDUCTION POTENTIALS

Zone of Visible Reduction

The zone of reduction of cream and skim milk may be moved up and down the potential : time curve at will by the addition of various quantities of dye. In Figure 3, curves A, B, C, and D are representative of many experiments made to determine this point.

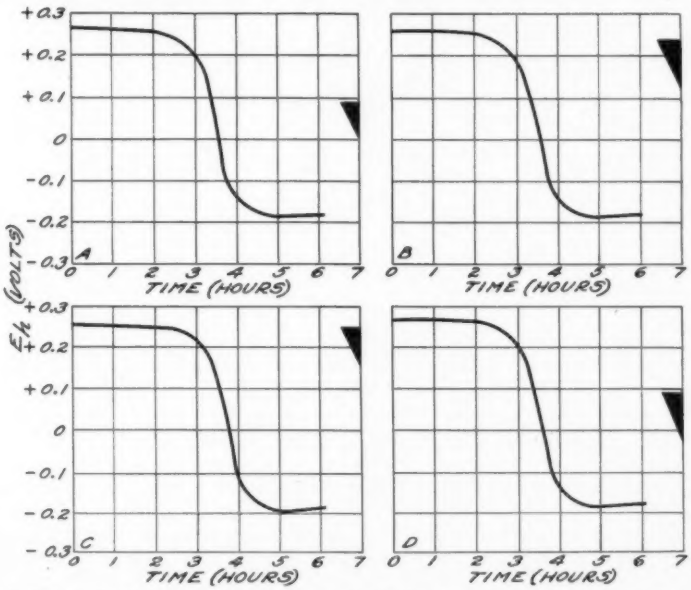

FIGURE 3.—Relation between the concentration of methylene blue used and the zone of reduction in skim milk and in 40 per cent cream: A, 1:200,000 of methylene blue in skim milk; B, 1:200,000 of methylene blue in cream; C, 1:16,000,000 of methylene blue in skim milk; D, 1:10,000 of methylene blue in cream. Potentials of the methylene blue zone of reduction are indicated by black triangles

Curves A and B show the zone of reduction of methylene blue in skim milk and cream, respectively, when the standard amount of dye (1:200,000) is added.

By adding only 1 part of dye to 16,000,000 parts of skim milk the zone was changed to approximate that of cream (B). Similarly, D shows that the addition of 1 part of dye to 10,000 parts of cream caused the zone of reduction to approximate the E_h limits which apply to skim milk when the standard amount of dye is added.

Reduction Time

In recent years there has been some controversy in regard to the effect of various concentrations of dye on the reduction time of milk. Three portions of a sample of milk containing the following concentrations of methylene blue were studied potentiometrically, (A) 1:400,000, (B) 1:200,000, and (C) 1:100,000. The potential:time curves of these three samples and the zones of reduction are shown in Figure 4. The three curves are so similar in form that they would almost superimpose if plotted upon the same ordinates. The zones of potential within which the methylene blue is reduced are shown by means of triangles. The position of these zones varies with the concentration of dye. In curve B, representing the sample containing the normal concentration of dye, decolorization took place in the zone between +0.225 and +0.165 volt, and was complete after 75 minutes of incubation. Curve A represents the sample containing

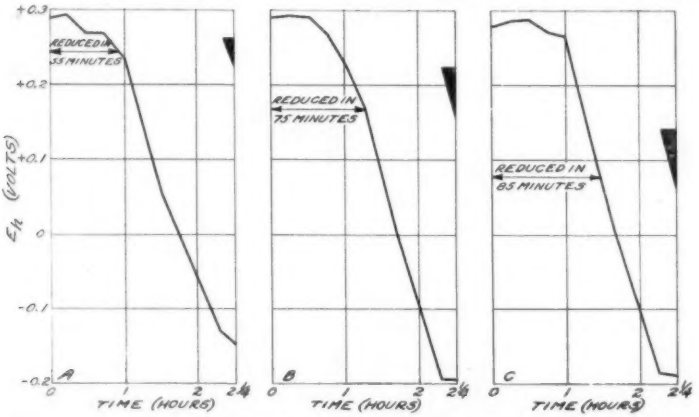

Figure 4.—Effect of varying the concentration of methylene blue in milk upon the reduction time: A, 1:400,000 of methylene blue; B, 1:200,000 of methylene blue; C, 1:100,000 of methylene blue. Potential of the methylene blue zone of reduction are indicated by black triangles

one-half the normal amount of dye (1:400,000). The zone of decolorization was 0.075 volt more positive than when the usual concentration of dye was used (curve B). Coincident with the more positive zone, the reduction time was shortened from 75 to 55 minutes. Curve C shows the effect of adding twice the usual concentration of dye (1:100,000). The zone of decolorization of methylene blue in this sample was 0.09 volt more negative than in the sample containing the usual concentration of dye. The time required for the reduction of the dye was increased to 85 minutes as compared with 75 minutes for sample B.

The significant aspect of these three potential:time curves is the potential of the zone of decolorization of the various concentrations of methylene blue. If it be assumed that the color disappears when less than an arbitrary minimum number of molecules of the blue dye are present, the explanation of the effect of the various amounts of dye on reduction time becomes simple. If more than the normal

amounts of dye are present, more negative potentials must be reached before decolorization is effected, and hence a longer time is required. Similarly, less time would be required to attain the slightly negative potential necessary to effect decolorization of sample A. (Fig. 4.) In other words, the more dye there is present the longer is the time required to reach a potential sufficiently negative to diminish the quantity of the dye in the oxidized form below the amount requisite for coloring.

In the concentrations employed (fig. 4) the dye does not affect the course of potential change, as is evidenced by the similarity of the three curves.

Form of Potential: Time Curve

The studies in the preceding experiments on the relationship of the concentration of dye to other factors were confined, for the most part, to higher dilutions of methylene blue. In the following experi-

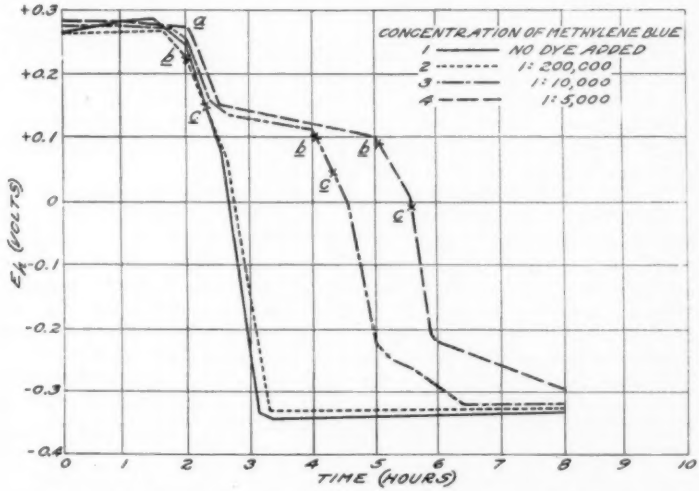

FIGURE 5.—Effect of varying the concentration of methylene blue in 20 per cent cream upon the reduction time of methylene blue

ment the effect of more concentrated solutions of dye has been studied. The curves in Figure 5 show the potential drift of four portions of a sample of 20 per cent cream containing the following concentrations of methylene blue: (1) No methylene blue; (2) 1:200,000; (3) 1:10,000; and (4) 1:5,000. The zones of potential within which the methylene blue reduced are indicated by the letters *b* (began) and *c* (completed). The curves of samples 1 and 2 are similar to those in Figure 4 and show that the addition of the normal amount of methylene blue does not alter the form of the potential: time curve. The zone of reduction of the dye in sample 2 was between the E_h values of +0.24 and +0.20 volt. The potentials of this zone are similar to those previously observed (fig. 4) for 20 per cent cream containing the standard amount of dye.

The potential curves of samples 3 and 4 illustrate clearly the effect of adding excessive amounts of methylene blue. There are several significant aspects of these curves which not only show the effect of the addition of excessive amounts of dye, but possibly throw some light on the mechanism of dye reduction in milk.

In the first place, all four samples began their swing toward the negative potentials simultaneously. Whether the initial fall in potential is directly or indirectly the result of bacterial activity, these results indicate quite clearly that at least the highest concentration of dye employed (1: 5,000) did not exert any antiseptic action.

The plateaus observed in curves 3 and 4, especially when contrasted with the total absence of a plateau in curve 2, emphasize the fact that the poising effect of the dye is directly dependent upon the amount of dye added. Clark (*6, p. 10*) defines poising as follows: "A solution may be said to be poised when it tends to resist a change in E_h on addition of an oxidizing or reducing agent."

As the potentials of the four samples of milk began their initial swing (point *a*) toward negative values, they followed the same general course until well within the zone of reduction of methylene blue. The potential drift was not impaired in sample 1 without dye, or in sample 2 in which the standard concentration of 1: 200,000 was employed. In samples 3 and 4, however, the large amounts of methylene blue added exerted a poising effect which was directly related to the quantity of dye added.

Since the usual concentration of dye employed in the reductase test does not materially affect the oxidation-reduction system, the methylene blue simply serves as a visible indicator that this swing toward more negative potentials has taken place. As the visible reduction occurs shortly after the swing toward more negative potentials begins, the loss of color of the dye indicates that the bacterial activity has overcome the poising action of the oxidation-reduction system or systems of the milk. (Point *a* has been reached.)

The time required for visible reduction became progressively greater as the concentration of dye was increased. For the samples reported in Figure 5 the dye concentrations and the reduction times were as follows:

(2) 1: 200,000—128 minutes.
(3) 1: 10,000—250 minutes.
(4) 1: 5,000—335 minutes.

The reduction of dye in samples 3 and 4 was not completed until after the second drop in the potential had started. The data in Figure 5 further substantiate the observations made in connection with Figure 3, viz, that the amount of dye employed affects the zone of reduction.

EFFECT OF THE CONCENTRATION OF SUGAR ON OXIDATION-REDUCTION POTENTIALS IN CREAM

There is a demand at the present time for a practical test to determine the sanitary quality of dairy products such as ice cream and ice-cream mixes. Early in the course of these experiments, attempts to follow the course of the potential drift of ice cream showed that the curve tended to pass slowly toward negative values. The visible reduction of the dye was correspondingly delayed over an

extended period. Attempts to determine the cause of the peculiar nature of the curve led to a series of experiments to demonstrate the effect of sugar on the potential drift. Figure 6 shows the results of a typical experiment.

Sterile cream, skim milk, and a cane-sugar solution were combined in suitable proportions to give various concentrations of sugar (0, 10, 20, and 25 per cent) and a constant fat content of 20 per cent. Each sample was inoculated with a 24-hour culture of *Streptococcus lactis* and the standard amount of dye was added. The form of the potential: time curves was markedly altered by increasing the percentage of sugar. Increasing the amount of sugar delayed the potential trend to more negative values, which in turn lengthened the reduction time. Although equal amounts of inoculum were added to each sample, obviously the number of bacteria added could not be accurately

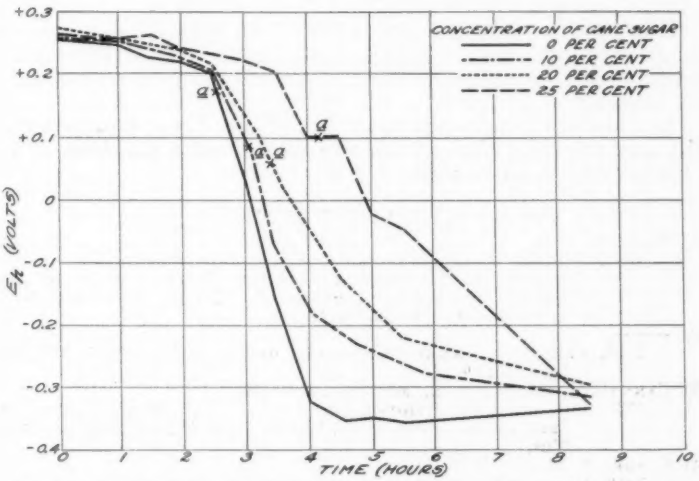

FIGURE 6.—Effect of varying the concentration of cane sugar in cream-skim milk ice-cream mixes upon the reduction time of methylene blue. Reduction occurred at point *a* on each curve. The fat content of the mix was 20 per cent

controlled. Nevertheless, the time required for reduction was increased directly with larger amounts of sugar. The reduction times for the samples in the order of increasing amounts of sugar were 155, 185, 205, and 248 minutes, respectively. The differences in the form of the potential: time curves may have been due to a change in the metabolic activities of the cells, although evidence to support this suggestion is not available. It has been shown by Hewitt (*20*) that changes in the medium affect the reduction intensities attained by bacterial cultures. It is of interest to note the extreme negative levels (−0.3 and −0.35 volt) attained by these cultures. Clark and his associates (*6*), Rogers and Whittier (*23*), and also Frazier and Whittier (*13*) have shown that cultures of *S. lactis* in milk usually reach a negative limit of approximately −0.2 volt.

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SUMMARY

The potential: time curves of milk with and without methylene blue remained in close agreement during the entire reduction process. The blue color and initial potentials of reduced samples could be restored by vigorous shaking or aspirating with air. Either of the above treatments also restored the initial potentials of samples without dye.

The bacterial flora of a sample of market milk influences the form of the potential: time curve.

The position of the zone of visible reduction was caused to vary by altering either the fat content of the sample or the concentration of the dye added. The zone of reduction became more positive with an increase in the percentage of fat and more negative with an increase in the concentration of dye.

The time required for visible reduction increased as the zone of reduction became more negative.

When excessive amounts of dye (1:10,000) were added, the potential of the solution did not pass smoothly to more negative limits, but was deterred as it approached the zone of reduction characteristic of this indicator.

The addition of cane sugar to cream not only delayed the potential drift and reduction time of the dye, but affected the form of the potential: time curve as well.

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EFFECT OF LIGHT ON THE REDUCTION OF METHYLENE BLUE IN MILK[1]

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INTRODUCTION

In a previous paper (*2*)[2] it has been shown that methylene blue in milk changes to methylene white when a certain specific zone of potential is reached. In the concentrations employed in the reductase test the dye apparently exerts no effect on the potential drift, and therefore serves merely as a visible indicator that a definite reducing intensity has been attained.

For a number of years it has been known that a medium such as milk or broth containing methylene blue loses its color when exposed to sunlight. It has not been determined, however, whether sunlight merely bleaches the dye directly or causes a change in the reducing intensity of the substrate comparable to that induced by bacteria. The purpose of studying the effect of light on the reduction of methylene blue was to establish more firmly the correlation between reducing intensities and dye reduction, and thereby afford a more intelligent approach to the fundamental mechanism of such reactions.

LITERATURE REVIEW

Gebhard (*5*), and Lasareff (*8*) have shown that the bleaching effect of light on methylene blue is most intense in the absence of oxygen provided the available light consisted of waves shorter than 620 μ; the color returned in the dark in the presence of oxygen.

In connection with his studies on anaerobiosis, Hall (*7*) observed that light induced the decolorization of methylene blue when added to broth cultures.

Whitehead (*11*) summarizes his work on methylene blue reduction in sunlight as follows:

> 1. Methylene blue added to fresh milk of good quality is reduced in a short time in the presence of sunlight at 37°. * * *.
> 2. The reduction in sunlight is not due to an enzyme * * *.
> 3. Milk from which the fat has been removed by centrifugal separation no longer gives the reaction, but the activity of the milk can be restored by an addition of sodium oleate. Sodium palmitate has not a similar action.
> 4. It is suggested that sunlight catalyses an oxidation-reduction reaction in which unsaturated fats are oxidised and methylene blue is reduced.

In a discussion of the theoretical aspects of oxidation and reduction of colored compounds Michaelis (*9, p. 69*) suggests that they may all have loose electrons which render them peculiarly adapted to oxidation-reduction systems in the presence of suitable electron acceptors. The decolorized dye likewise may acquire electrons from still more powerful reductants. These properties not only facilitate their rôle in reversible oxidation-reduction systems, but may explain the fre-

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[2] Reference is made by number (italic) to Literature Cited, p. 95.

quently observed catalytic action of such compounds in oxidations. Michaelis further suggests that although these theories are not well established they may account for the effect of radiant energy on the instability of electrons.

METHODS

In order to correlate the reduction of methylene blue in milk by light and the changes in reducing intensity, samples of milk were subdivided so as to give replicates with and without dye, exposed, and unexposed to light. The potential drifts in the various samples were followed by means of the apparatus described in a previous paper (*2*). In order to afford a proper exposure to the sun's rays test tubes containing the samples were submerged to a depth of about 1 inch in a water bath maintained at a suitable temperature. Because of the prevailing low outdoor temperature during most of the experiments it was not feasible to place the water bath in an open window, and

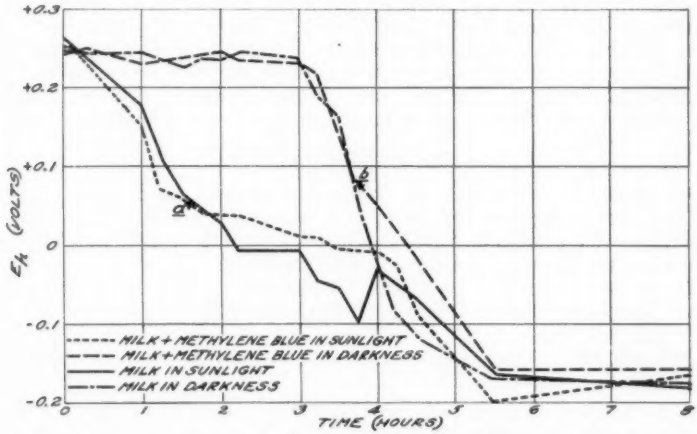

FIGURE 1.—Effect of sunlight on the potential : time curves and reduction time of market milk with and without the addition of methylene blue, complete reduction took place at points *a* and *b*

therefore the sunlight filtered through a window pane in addition to the walls of the test tube. It may be assumed that the effective light did not contain rays shorter than approximately 340 μ; the value usually given for the transmissibility of ordinary glass.

EXPERIMENTAL DATA

EFFECT OF SUNLIGHT ON THE OXIDATION-REDUCTION POTENTIAL OF MARKET MILK, CREAM, SKIM MILK, AND SKIM MILK PLUS SODIUM OLEATE AND SODIUM STEARATE

It was consistently observed that in the tubes exposed to sunlight the dye was reduced within 15 to 90 minutes, the length of time depending upon the intensity of the sunlight. It was also generally observed that increasing the percentage of fat shortened the time required for reduction of the dye. Similar results were obtained by adding increasing amounts of sodium oleate to skim milk. In order to obtain a more complete history of the changes occurring in milk,

cream, and skim milk exposed to sunlight, the oxidation-reduction potentials of a number of samples were measured at suitable intervals.

Market Milk

Ten cubic centimeter samples of market milk were placed in each of four sterile test tubes and the standard amount of methylene blue (1: 200,000) was added to two of them. One tube of milk containing methylene blue and one without dye were placed in the sunlight; the two remaining tubes were covered with a sleeve of heavy black paper.

Figure 1 shows that the potentials of the samples exposed to sunlight became more negative immediately after exposure. This negative drift continued until an E_h value of approximately zero was reached. The milk containing the methylene blue was completely reduced at an E_h value of $+0.065$ volt (point *a*). After the initial rapid fall the potentials remained at an E_h value of approximately zero for three hours, or until four hours after the beginning of incubation. At this time the potentials of the four tubes came into close agreement. The potentials of the two tubes not exposed to sunlight had retained their initial E_h values for three hours, at which time they began to fall rapidly to the negative side. The potential drift of the milk in the dark may well be attributed to bacterial activity.

Gillespie (*6*) first suggested that bacterial cultures may induce a negative drift of electrode potential. Clark and his associates (*1*), Thornton and Hastings (*10*), and Frazier and Whittier (*3*, *4*) have studied the effect of bacterial cultures upon the electrode potential of milk.

Attention is called to the fact that the four curves (fig. 1) tend to converge at an E_h value of approximately zero. This is considerably more positive than the ultimate negative limit of the potential drift (-0.2 volt). A comparison of these curves shows quite clearly that the light was unable to lower the potential below the E_h value of approximately zero. After these curves converged with those of the two tubes kept in the dark, all remained in close agreement throughout the remainder of the reduction process. The visible reduction of the sample in the light (*a*) preceded that of the one in the dark (*b*) by 2.5 hours.

The samples in the dark and in the light showed complete visible reduction (points *a* and *b*) at approximately the same E_h value. This suggests that the reduction of the standard quantity of methylene blue (1: 200,000) in milk takes place within a definite potential zone, and that the change of color occurs whenever this potential is reached, whether the potential drift be induced by physical or biochemical processes. Parallel determinations of the hydrogen-ion concentration of samples exposed and protected from the light indicate that the variations in potential drift could not be accounted for on a basis of changes in pH.

Cream

The effect of sunlight on the oxidation-reduction potentials of four portions of a sample of 40 per cent cream with and without dye were studied in the same manner as in the preceding experiment. The potential : time curves are shown in Figure 2. As in the case of milk, sunlight induced a negative potential drift immediately after exposure, whereas the E_h value of the samples in the dark remained con-

stant for several hours. In this experiment, however, the presence of higher concentrations of fat apparently affected the ability of the light to induce more negative E_h values. The initial potential drift in the two samples (with and without dye) exposed to sunlight is extended over a considerable period, in contrast to the rather sudden fall observed for whole milk. (Fig. 1.) Also, a comparison of curves for the samples exposed to sunlight shows that when methylene blue is present sunlight is able to induce more negative potential values than when no dye is added to the cream.

As in the preceding experiment, the potentials of the zones of visible reduction (*a* and *b*) are essentially the same for samples in the light and in the dark. The presence of fat tends to elevate the zone of reduction, whether induced by bacterial action or sunlight. The fact that increasing percentages of fat shorten the reduction

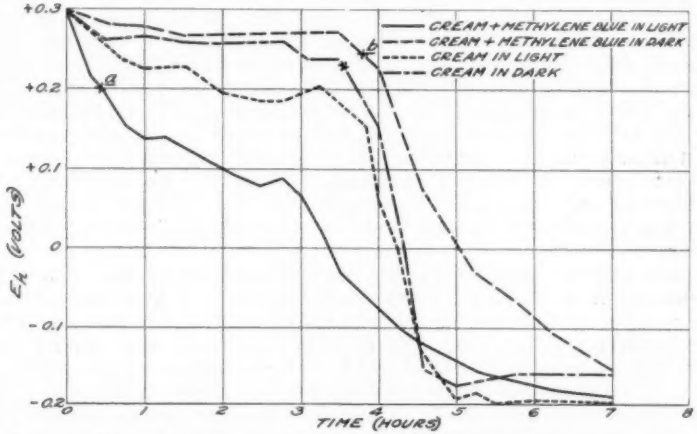

FIGURE 2.—Effect of sunlight on the potential: time curves and reduction time of cream (40 per cent fat) with and without the addition of methylene blue; complete reduction took place at points *a* and *b*

time of exposed samples is apparently due to the tendency of fat to elevate the zone of reduction.

SKIM MILK

The oxidation-reduction potentials of skim milk with and without dye exposed to the light and in the dark were followed in exactly the same manner as for cream and whole milk. The potential: time curves of the samples of skim milk are shown in Figure 3. The curves are similar in a general way to those presented in Figures 1 and 2 for whole milk and cream. However, sunlight causes a greater and more rapid fall of potential in the skim milk than in either cream or whole milk. Nevertheless, the lower zone of reduction in the skim milk lengthens the time required to attain the reducing intensity necessary for complete loss of color. As in the case of cream, the addition of dye to the skim milk enabled the sunlight to induce a more negative potential drift than in the same skim milk without dye. In harmony with previous observations, as shown in Figures 1 and 2, the E_h

values at the time of complete visible reduction (*a* and *b*) were essentially the same, whether induced by bacteria or sunlight. The potentials of the four samples came into close agreement after 3.5 hours and remained together during the remainder of the reduction process.

Skim Milk Plus Sodium Oleate and Sodium Stearate

In the preliminary studies on the reduction of methylene blue by sunlight, it was observed that skim milk containing sodium oleate and methylene blue was readily reduced. In order to determine the effect of such substances on the potential of the zone of reduction of methylene blue, a sample of skim milk was divided into three parts and treated as follows: (1) 1 per cent sodium oleate, (2) 1 per cent sodium stearate, and (3) not treated. The standard amount of methylene blue was added to each of the three samples. The potential: time curves and points of complete visible reduction of the three

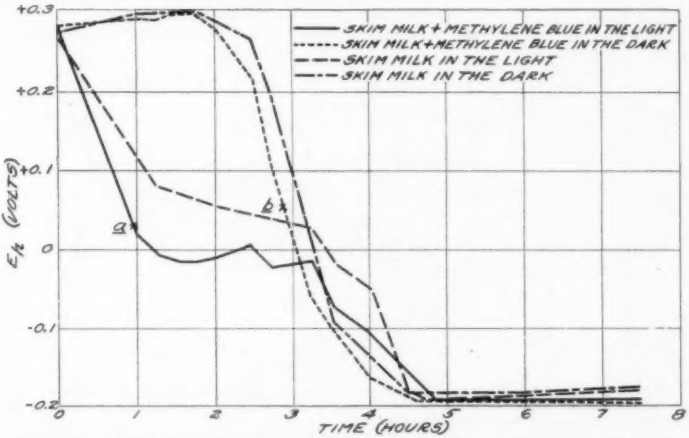

FIGURE 3.—Effect of sunlight on the potential: time curves and reduction time of skim milk with and without the addition of methylene blue; complete reduction took place at points *a* and *b*

samples are shown in Figure 4. The potentials of the samples containing the fatty acid salts drifted toward the negative side more rapidly than they had in the case of skim milk. The potentials of these two samples dropped rapidly to $E_h - 0.025$ volt, after which they remained fairly constant. After the samples had incubated for four hours no more sunlight was available and the potentials returned to the positive side. The potentials remained in close agreement throughout the remainder of the reduction process. After seven hours the potential of each sample quickly drifted to the negative limit of approximately -0.2 volt, induced, no doubt, by bacterial activity.

The letters *a*, *b*, and *c* on Figure 4 represent the points at which the methylene blue was completely decolorized. The zone of reduction of methylene blue is evidently not affected by the presence of either of the fatty acid salts employed in this experiment. Not only does the uniformity of the E_h values at the time of reduction of the

dye in the three solutions (*a*, *b*, and *c*) emphasize this fact, but the values conform to those previously observed for the zone of reduction of this dye in skim milk (approximately −0.025 volt). Any deterring influence which butterfat may have exerted on changes in potential in the preceding experiments, apparently is not induced by 1 per cent of sodium oleate or sodium stearate.

It was observed in a preceding experiment (fig. 2) that the presence of butterfat shortens the reduction time of exposed samples by elevating the zone of reduction. The tendency of sodium oleate and sodium stearate to shorten the reduction time is apparently due to their ability to accentuate the potential drift. The contrast between the action of butterfat and these readily oxidizable substances may throw some light on the mechanism of dye reduction.

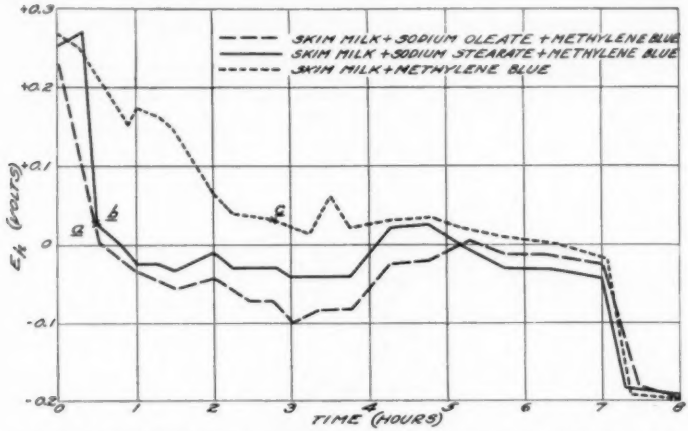

FIGURE 4.—Effect of sunlight on the potential: time curves of skim milk, skim milk plus sodium oleate, and skim milk plus sodium stearate, with the addition of methylene blue; complete reduction took place at points *a*, *b*, and *c*

EFFECT OF CONCENTRATION OF DYE ON THE OXIDATION-REDUCTION POTENTIAL

The results of previous experiments indicate that the presence of methylene blue accelerates the potential change in cream and skim milk when these solutions are exposed to sunlight. In order to study more fully the rôle played by methylene blue in this reaction, skim milk containing 1 per cent sodium oleate was divided into six parts, and methylene blue was added as follows: (1) None, (2) 1:400,000, (3) 1:200,000, (4) 1:100,000, (5) 1:50,000, (6)1:25,000. The tubes were placed in the water bath and exposed to sunlight. The potential curves and points at which visible reduction was completed are presented in Figure 5.

The potentials of all the samples not only drifted to more negative values when the tubes were exposed to sunlight, but except in the case of sample 6 (1:25,000), the fall of potential was directly related to the concentration of dye. The potential of sample 6 did not reach the negative limits attained by samples 4 (1:100,000) and 5 (1:50,000). The most marked difference observed was between samples 1 (no dye) and 2 (1:400,000). It is quite evident that the presence of only a small quantity of dye greatly accentuates the

potential change induced by sunlight. The accelerating action of the dye is not directly proportional to the amount of dye added. The addition of methylene blue in concentrations higher than 1 : 200,000 did not materially increase the reducing intensities induced by sunlight. The potentials of all the samples remained fairly constant after the intitial drift toward the negative side. After the samples had incubated for five hours the potentials dropped rapidly to more negative limits. These latter changes in potential are due, no doubt, to bacterial activity.

In Figure 5 it may be observed that the final negative limits attained by the samples, with one exception, are inversely related to the concentration of dye added. The exception noted is sample 1, the negative limit of which is slightly more positive than that of sample 2. The time required for visible reduction in the six samples

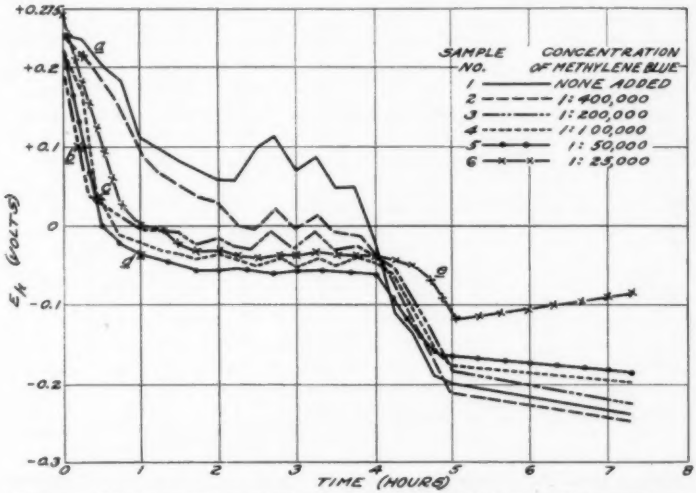

FIGURE 5.—Effect of sunlight on the potential : time curves of skim milk containing various concentrations of methylene blue when exposed to sunlight; complete reduction took place at points *a* to *e*

increased directly with the amount of dye added. The time varied from 15 minutes for sample 2 (1:400,000) to 285 minutes for sample 6 (1:25,000). The E_h values at which visible reduction of the dye was complete (*a*, *b*, and *c*) became more negative with each increase in concentration. Sample 6 was not completely reduced by the sunlight. Though lighter in shade, some color was still discernible at the time sunlight was no longer available. Decolorization of this sample was effected only after bacterial action had induced a more negative reducing intensity.

EFFECT OF ALTERNATE LIGHT AND DARKNESS ON THE OXIDATION-REDUCTION POTENTIAL

Figure 6 illustrates the change in oxidation-reduction potentials induced by alternately placing a solution in the light and in the dark. Samples of skim milk and market milk were each divided into two portions and methylene blue (1:200,000) added to one of each.

These four samples were exposed to sunlight, and when the potential of a sample had drifted toward more negative values the sample was covered with a sleeve of heavy black paper to exclude the light. The effect on the potential of alternately placing milk or skim milk in the light and dark is illustrated in Figure 6. At the points on the curves labeled *d* the samples were placed in the dark, and at points *l* they were again exposed to light. The potential of the sample of skim milk plus methylene blue dropped quickly to E_h +0.08 volt at the beginning of the experiment; when the sample was placed in the dark the potential rapidly returned to the more positive E_h value of +0.2 volt. When the sample was again placed in the light the potential drifted quickly back to an E_h value of approximately +0.1 volt. After the sample had been exposed to three hours of incubation, the sun, although still shining, had disappeared behind adjacent buildings, thereby diminishing the intensity of the effective light. Skim milk

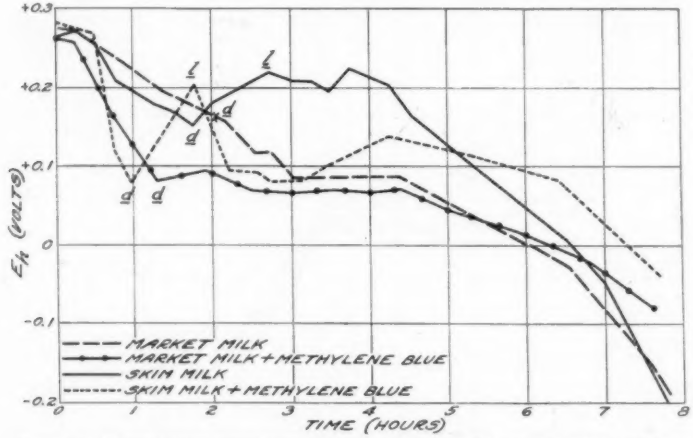

FIGURE 6.—Potential : time curves of market and of skim milk, with and without the addition of methylene blue, when placed alternately in sunlight and in the dark; the samples were placed in the dark at points indicated by *d* and in sunlight at points indicated by *l*

containing methylene blue (1:200,000) was consistently found to be very responsive to any diminution of light intensity, as is evidenced by the slight drift in potential between the third and fourth hour of experiment. Effective sunlight was no longer available after the fourth hour of this particular experiment.

The potential curve of skim milk without methylene blue shows that alternate placing of the sample in the light and in the dark affects the potential drift. However, the response of the electrode potential to light is not as great as in the case of skim milk plus dye.

The potential curves of market milk with and without dye show that the potentials drift to more negative values when exposed to sunlight, but do not return to more positive values when placed in the dark. The sample of market milk when placed in the dark not only failed to respond by swinging to more positive values, but continued its uninterrupted negative potential drift.

These observations suggest: (1) That methylene blue accentuates the response of the electrode potential to the effects produced by the

presence or absence of light, and (2) the presence of fat has a deterring influence on the potential drift as induced by light. This latter observation is in harmony with those made in connection with Figures 1 and 2.

The complexity of the oxidation-reduction system or systems in milk and cream renders hazardous any attempt to speculate on the probable mechanism of light in these observations. The trend toward more negative potentials when milk is exposed to light suggests, however, the participation of some ingredient which is temporarily oxidized while under the influence of light, and which regains its electron at the expense of the electromotively active system as soon as the effect of the light is removed.

It is possible that the deterring influence of fat on the reversibility of this process may be due in part to the sluggishness of electrodes in the presence of increasing amounts of fat. However, if the effect were entirely apparent rather than real—that is, if increasing percentages of fat affected only the electrode sensitivity—one would expect a return of the blue color when previously exposed cream was returned to the dark. Since the failure of the electrode to record a trend toward positive values is corroborated by a failure of the blue color to return when cream which has been exposed to the light is returned to the dark, it suggests that the fat exerts an actual deterring influence on the reversibility of the reaction. Whether it serves as a shield or protector of the electromotively active oxidation-reduction system or actually participates in the sharing of electrons can not be deducted from the observations at hand.

EFFECT OF ARTIFICIAL LIGHT ON THE OXIDATION-REDUCTION POTENTIAL

It is a common practice in the determination of quality of milk by the methylene blue reduction test to incubate the samples in a constant temperature incubator. The temperature of such incubators is usually regulated by using electric lights as a source of heat. It has been observed that when samples are incubated in this manner those nearest the light are reduced in the least time.

In a preliminary experiment samples near an electric light were reduced 2.5 hours earlier than shielded samples. In order to determine the cause of this difference in reduction time the following experiment was conducted. A sample of market milk of good quality was divided into four parts and the standard amount of methylene blue was added. The samples were placed in an incubator maintained at 37° C. by two 75-watt bulbs. One of these burned constantly and the other was operated intermittently by the thermostat. Duplicate tubes of milk were protected from the light by sleeves of heavy black paper and two others were exposed to the light rays from the electric bulbs. Representative potential curves and reduction times of two of these are presented in Figure 7.

An examination of these curves shows that artificial light affected the potentials and reduction time in much the same manner as was observed in the case of sunlight. The potential of the exposed sample drifted slowly toward the negative side, whereas that of the shielded sample remained fairly constant for six hours. As shown in Figure 7, the exposed sample was completely reduced about 2.5 hours sooner than the shielded sample.

Temperature was not a factor in hastening reduction of the exposed samples. Since the temperature of the exposed samples was 1° C. lower than that of the ones in the dark, it is not possible to attribute the more rapid reduction to this factor.

SUMMARY

The potentials of cream, whole milk, and skim milk drifted toward the negative side when these solutions were exposed to sunlight. Potential changes to both more positive and more negative values were deterred by the presence of fat. This influence exerted by fat was especially noticeable when solutions were alternately placed in the sunlight and in the dark. The addition of fat to skim milk hastened the reduction time of methylene blue in samples exposed to the sunlight, owing probably to the tendency of fat to elevate the zone of reduction. Sodium oleate and sodium stearate also shortened the reduction time, but did so by causing a more rapid fall of potential.

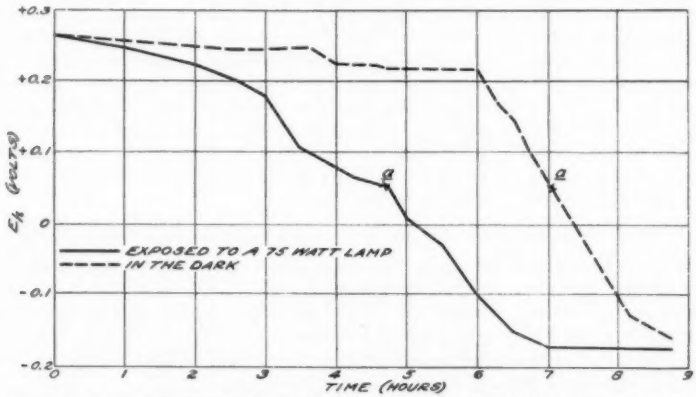

FIGURE 7.—Effect of exposure to a 75-watt electric light upon the potential : time curves and reduction time of market milk; complete reduction took place at point *a*

The addition of methylene blue to skim milk or cream accentuated the potential changes induced by sunlight. With each increase up to 1:25,000 in the concentration of dye added to skim milk containing sodium oleate, the reducing intensities induced by sunlight were progressively more negative.

Visible reduction of methylene blue induced either by sunlight or bacterial activity took place within the E_h limits characteristic for the particular sample.

The reducing intensity induced by bacterial activity was more negative than that induced by sunlight. In the case of sunlight the negative limits reached were seldom below zero, as compared with a reducing intensity of -0.2 volt induced by bacteria.

These observations confirm Whitehead's conclusion, that reduction of methylene blue by light is a reaction distinct from the reaction induced by bacteria.

It was observed that as the solution developed a progressively more negative potential the methylene blue decolorized whenever this potential passed through the zone of reduction characteristic of

this dye. Similarly, the blue color reappeared when the solution developed a potential sufficiently positive to oxidize the dye present. When skim milk plus methylene blue which had been reduced by sunlight was placed in the dark, the potentials quickly became sufficiently positive to oxidize the dye.

Artificial light hastened the reduction of methylene blue in market milk. Light from a 75-watt electric lamp induced a potential drift in milk which differed only in degree from that observed in the case of sunlight. The reduction of methylene blue in one sample of milk was hastened 2.5 hours by exposure to light from an electric bulb.

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